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255. A STUDY OF PERSISTENCY IN A HERD OF AYRSHIRE COWS

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(With 1 Figure)

ALTHOUGH the endocrine mechanism of lactation in the cow is incompletely understood, it can be provisionally outlined thus (Gaines (1), Gaines & Davidson (2), Turner *et al.* (3, 4), Espe (5)). During pregnancy the secretion of oestrogen through anterior hypophyseal hormones induces the development of the secretory system of the udder. At parturition the high secretion of anterior hypophyseal lactogen (or the removal of some inhibitory or inactivating cause) starts and rapidly raises the production of milk. The peak is reached at a time, varying with individuals and with age, from a few days up to 70 or more after parturition. From this peak the rate of production gradually decreases, generally following a curve with slight upper concavity. The cause of this decline is obscure: it might be due to a reduction in the output of lactogen or to senility of the alveoli or more probably to both causes; the effectiveness of lactogen being possibly dependent not only on its amount but also on the presence of alveolar cells capable of responding to its stimulus. The effects of a new pregnancy on the slope of the lactation curve is not exactly known: however, the first four or five months of pregnancy have no appreciable influence. Later on a somewhat stronger effect occurs, perhaps connected with the rise in the output of oestrogen as pregnancy proceeds. Oestrogen would then seem to depress the milk secretion of the current lactation, but it has undoubtedly a function in the preparation of the udder for a new one.

In the first part of the lactation the stimulus to produce milk is so strong that the environment, especially the plane of nutrition, can but slightly and only temporarily affect it. The contrary is true as lactation proceeds.

As pointed out by Bonnier (6), the first procedure in trying to find an appropriate measure of the decline in the rate of milk production, is to establish *what* portion and how *large* a portion of the lactation curve is to be employed.

While for practical purposes in animal breeding—as when assessing the persistency of different cows for registration, selection, etc.—a single figure describing the total trend of the lactation is desirable, for genetical and physiological investigations a subdivision of the lactation curve appears necessary in view of its complexity. It seems logical to divide the curve, on

the basis of the supposed endocrine mechanism already outlined, in the following manner:

(1) *The rapidly rising portion* after calving, the trend of which can be considered as the combined result of the development in the glandular system *before* parturition (connected with oestrogen output) and of the intensity of the stimulating factors *after* parturition.

(2) *The declining central portion*, connected with the ageing and wearing away of the glandular tissue, perhaps associated with a reduction in the stimulation.

(3) *The rapidly declining terminal part* (drying off), the causes of which are unknown. As a working hypothesis it might be supposed that this final drop occurs when one, or both, of the factors under (2) has reached a certain lower threshold.

The effect of a new pregnancy has already been pointed out. It appears that this effect is slight up to 5 months after service (i.e. approximately 8 months after freshening), but later on probably an acceleration in the rate of decline of the curve occurs. The effect of environment (chiefly nutrition) becomes greater as the lactation advances and is decisive in the final part.

The aim of the present work has been to study the central portion of the lactation curve in a particular herd in which there is evidence that management has not appreciably changed in the 18 years considered. This central portion of the curve defined as the one, generally with feeble upper *concavity*, lying between the peak after parturition and the final drop (upper *convexity*), is almost independent of the overlapping effect of a new pregnancy and is affected by environment to a lesser extent than the final portion.

The other two portions are not necessarily of minor importance. In particular the final one—which shows great variation both in the point at which it starts (i.e. the curve becomes steeper and with upper convexity), and in its trend—is that which mainly determines the *actual* length of the lactation.

In the present work the term persistency is applied only to the central part of the curve, as already defined, of which it expresses in some way the steepness. Among the measures of persistency, the one illustrated by Gaines (7) and later adopted by Gooch (8) and others has been chosen.

It is based on fitting an exponential $y_t = y_0 e^{kt}$ (in the present work $y =$ pounds of milk per day, $t =$ time from calving in 30 days units) to the lactation curve. The measure of persistency is given by e^k which *approximately* expresses the ratio of the daily yield, in each 30-day period, to the daily yield in the preceding one. It therefore expresses persistency as a constant *proportional* monthly reduction in yield from the initial yield y_0 (theoretical value of y for $t = 0$). As e^k, which shall be denoted as P, is a *proportional* measure of the slope, the values found in lactation curves differing in initial yield are, other conditions being equal, comparable. Fig. 1 gives in arithlog scale a lactation curve of high persistency ($P = 0{\cdot}93$) and one of low persistency ($P = 0{\cdot}80$). The two heavier straight lines are those fitted by least squares to the central portions

of the curves. The initial and final portions, not used in the interpolation, are in dotted lines. It may be observed that although the two lactations (which started in the same part of the year) differ widely in maximum yield and in persistency, their actual length, and the total milk yields are approximately the same. This emphasizes the practical importance of the trend of the two other portions of the curve, and particularly of the final one.

It has been shown by Gooch (8) that initial yield and persistency are negatively correlated. This might be a consequence of the already mentioned double action of oestrogen and associated hormones during pregnancy, i.e. a depression in the current lactation and a preparation of the udder for the following one. In general, then, a high oestrogen output during pregnancy would probably be associated with a high initial yield and a rapid decline in

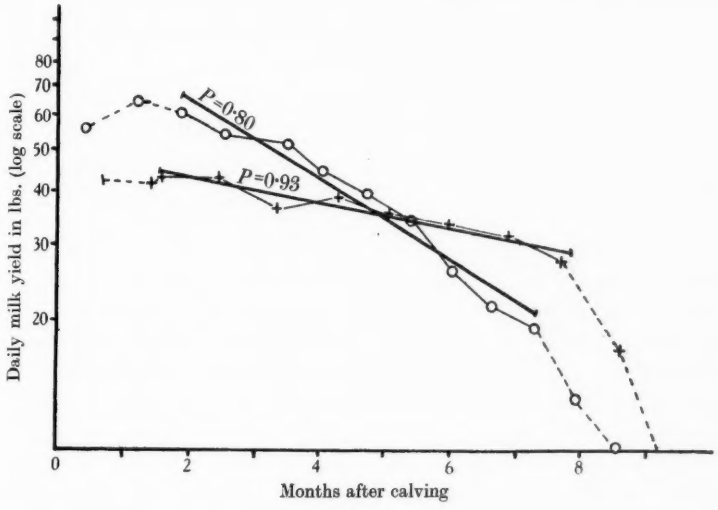

Fig. 1.

the latter part of the lactation curve. These are the characteristics of a low persistent cow (high maximum and steep decline). In other words it seems possible that, other things being equal, low persistent cows have a greater oestrogen secretion during pregnancy. This hypothesis could be checked by direct comparison of urine oestrogen in cows of low and high persistency and also by determining whether or not a new pregnancy has a greater depressing effect in cows with low persistency than in others. Unfortunately, our data are not suitable for this second relatively simple, though only preliminary, check.

The data for this study were obtained from a pedigree Ayrshire herd located in the south-west of Scotland and founded about 1920. They cover the period 1920–38. The foundation stock was made up with pure-bred recorded animals from different herds. They were practically unrelated and not inbred.

In the 18 years very few cows were introduced from outside, and they left no trace in the herd. All the bulls were home-bred except one, which, however, bore some relationship to two of the foundation animals. Practically no other outside "blood" entered the herd which, though maintained only at about twenty cows, has not shown inbreeding to a marked degree.

Table 1. *Inbreeding (Wright's method) in the cows with a second lactation in different periods*

Period	Average inbreeding coefficient %
Foundation cows	Trace
Cows with a second lactation in 1923–8	0·24
,, ,, ,, 1929–33	5·84
,, ,, ,, 1934–7	9·34

Table 2. *Relationship (Wright's method) between all the cows with a second lactation in different periods*

Period	Average *inter se* relationship coefficient %
Foundation cows	1·84
Cows with a second lactation in 1923–8	18·2
,, ,, ,, 1929–33	26·7
,, ,, ,, 1934–7	24·6

Thus, though the degree of inbreeding attained is rather low, as compared for instance with 26 % for the average of the Dairy Shorthorn breed in 1920 (Wright(9)),[1] the degree of relationship between cows is high, being approximately that of half sisters (25 %). This is a consequence of not having split the herd in separate lines. Somewhat severe selection has been practised for total butterfat and for milk yield. On the other hand, a rough comparison of the cows culled after the first lactation with the others, has given the impression that very little, if any, selection has been made for *persistency* as already defined. Its slight increase in the herd seems therefore attributable to the breeding policy (selection by pedigree) and not to selection based on individual performance.

A preliminary examination showed, in agreement with the findings of other authors, that the persistency of the first lactation is higher and has a greater variability than that of subsequent lactation. From the second lactation onwards no very definite trend has been observed. It was therefore decided to discard the first lactation from this study, and only cows with records of the second and successive lactations were taken into consideration. Eliminating in addition all the abnormal lactations (abortion, mastitis (when known), foot-and-mouth disease, etc.) a total of 163 lactations were available from fifty-nine cows, of which forty-one had, after the excluded first lactation, two or more records, and eighteen only one.

[1] Fowler(10), however, found the average for the Ayrshire breed as a whole to be only 4·6 %.

The curve $y_t = y_0 e^{kt}$ was fitted by least squares[1] to the recorded data (daily milk yield in pounds recorded approximately every 4 weeks) excluding the first part of the lactation (rising daily yields) and the terminal one. The determination of the latter is of course rather arbitrary: for long lactations, their records after 270 days were excluded; for the others, the fitting was stopped where the differences between the logarithms of successive yields began to increase. In most cases, the limits of the portion of the curve thus considered were about 50 and 240 days. The expression $e^k = P$, based on periods of 30 days, is taken as the measure of *persistency* in the limited sense already explained. In the following calculations P is multiplied by 100.

Table 3. *Statistics of P in* 163 *lactations* (*first excluded*) *from* 59 *cows*

Mean	$88{\cdot}42 \pm 0{\cdot}36$
Variance	21·37
σ	$4{\cdot}62 \pm 0{\cdot}26$
C. of V.%	$5{\cdot}22 \pm 0{\cdot}28$

The distribution of P is fairly normal. The variability of P, as shown by the coefficient of variation, appears to be rather low. This being in agreement with the findings of other authors in different conditions and with different breeds, the conclusion can be drawn that P is one of the characteristics of the lactation showing the least variation.

No definite effect of age was observed on P, after the first lactation. Excluding, however, the lactations over the 7th, 127 P's were chosen from the forty-one cows with two or more lactations (first excluded) and tested for the variance *between mean values of P for different cows* and *between values of P from the same cow but for different lactations.*

The results are as follows:

Table 4. *Analysis of variance of P*

Source of variation	Degrees of freedom	Sum of squares	Mean square	σ
Total	126	2946	23·381	4·84
Between means of cows	40	2001	50·025	3·52
Between lactations of same cow	86	945	10·988	3·52

The differentiation of the cows as regards P is highly significant and accounts for $\frac{23{\cdot}38 - 10{\cdot}99}{23{\cdot}38} = 52\ \%$ of the total variance. The remaining 48 % arises from variation of P between lactations of the same cow.

As the month of calving has been found by Gooch (8) and Sanders (11) to affect appreciably the shape of the lactation curve, and as there is a tendency for a cow to have successive calvings in approximately the same part of the year, it was thought that possibly part of the differentiation between cows could arise from this tendency. The mean P for each month was therefore

[1] The writer wishes to express his thanks to Dr A. C. Aitken, F.R.S., for his most helpful advice on this point.

calculated, and a proportional correction, based on the graphical interpolation of the curve relating P with month of calving, was applied.

Table 5. *Effect of month of calving on P*

Month of calving	Mean P found	Interpolated mean P	Correction factor for month of calving
January	89·36	89·4	0·990
February	89·26	88·7	0·998
March	86·62	86·7	1·021
April	84·41	85·4	1·036
May	85·79	85·0	1·041
June	84·33	85·2	1·039
July	87·04	87·0	1·017
August	89·61	89·2	0·992
September	90·24	90·6	0·977
October	92·51	92·0	0·962
November	92·91	92·3	0·959
December	89·96	90·7	0·976

The general trend of the effect of month is in agreement with the findings of Gooch, though naturally the *actual* effect of each month is different owing to different geographical conditions.

As is well known to breeders, persistency increases with autumn calvings, while with spring calvings it decreases. This is due to the high initial yield for spring calvers, followed by adverse nutritional conditions at the end of lactation (winter), and vice versa for the autumn calvers.

The use of a proportional correction is undoubtedly questionable, as it assumes, without proof, that cows respond to the seasonal variations in proportion to their P. It was, however, adopted *faute de mieux* and the results seem satisfactory, as shown by the following new analysis of variance of corrected P for the 127 lactations already dealt with:

Table 6. *Analysis of variance of P after correction for month of calving*

Source of variation	Degrees of freedom	Sum of squares	Mean square	σ
Total	126	2002	15·888	3·98
Between means of cows	40	1311	32·775	2·80
Between lactations of same cow	86	691	8·035	2·84

The correction for month of calving reduced significantly the variance of P, from 23·38 to 15·89, which shows that month of calving determines in this herd at least one-third of the variance. The reduction is more pronounced in the variance *between cows* than in that *between lactations of the same cow*, as might be expected from the tendency for cows to have successive calvings in the same season of the year.

Thus, after correction, the differentiation *between cows* accounts for $\frac{15{\cdot}89-8{\cdot}04}{15{\cdot}89}=49\ \%$ approximately of the corrected variance. Undoubtedly P is therefore in great part an expression of individuality: the physiological causes determining the repeatability of P in the different lactations of the same

cow are, in the group of cows studied, approximately of the same importance as those which tend to diversify P from one lactation to another. Of course, the latter cannot be considered as purely environmental (in the broader sense), because they include also errors of technique in the determination of P, both in milk recording and in fitting the curve to the recorded data.

Averaging several corrected P's from different lactations of the same cow tends to eliminate part of the variation due to environment and to technique, thus giving a better estimate of what might be called the inherent persistency of a cow. The accuracy of this estimate increases with the square root of the number of lactations available:

Table 7. *Standard error of the mean of corrected P from different lactations of the same cow (lactations considered: second to seventh inclusive)*

No. of lactations averaged. n	S.E. of the mean $2{\cdot}84/\sqrt{n}$
1	2·8
2	2·0
3	1·6
4	1·4
5	1·3
6	1·2

As the effect of age on P (the first lactation having been excluded) is certainly small, it is believed that the elimination of lactations beyond the seventh practically disposes of this factor. In conclusion, an average of P from three normal lactations, falling between the second and the seventh, gives a fairly good estimate of persistency as herein defined.

As a basis for investigating the inheritance of P in this herd, an average corrected P was taken for each cow from all available lactations between second and seventh.

A dam-daughter correlation was then calculated, making allowance for inbreeding by taking only the pairs in which the dam-daughter relationship coefficient did not exceed 0·60. (Without inbreeding the parent-offspring relationship is 0·50.) There were thirty-one pairs satisfying this condition and the correlation found is $r_{po} = 0{\cdot}166 \pm 0{\cdot}17$.

Owing to the limited number of individuals no further investigation on the relative importance of heredity and environment in the determination of persistency was attempted with this material.

SUMMARY AND CONCLUSIONS

Connecting the trend of the different parts of the lactation curve with what is believed to be the underlying hormonal mechanism, it has been pointed out that any analysis of the curve must deal separately with three parts. The central portion, lying between the peak of daily production and the rapid decline (drying off) has been the object of investigations in an inbred Ayrshire herd. Coefficients of persistency were obtained by fitting exponential curves to this central portion. When first lactations were excluded and correction for

month of calving applied, the coefficients (P) were found to be reasonably consistent for different lactations of the same cow. Month of calving accounted in this herd for about one-third of the variance of P. Of the remaining variance approximately one-half is attributed to differences among cows. A possible physiological explanation of the cause of the well-known negative correlation between maximum yield and persistency has been suggested. No conclusions can be drawn from this material on the relative importance of heredity and environment in determining persistency.

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256. THE PROTEIN REQUIREMENTS FOR MILK PRODUCTION

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(With 1 Figure)

The literature dealing with the protein requirements of animals is very extensive and, as most of this has been dealt with in recent reviews such as those of Mitchell(1,2), Halnan(3), Morris(4) and M'Candlish(5), it is unnecessary to elaborate the subject here.

In 1925 a departmental committee of the Ministry of Agriculture(6) recommended as the protein standard for milk production 0·6 lb. protein equivalent[1] per 10 lb. milk of 3·7 % fat. The main object of the present investigation was to decide whether, under practical conditions, this standard is unnecessarily high.

Methods

Large-scale experiments were carried out on a number of farms during the two winters 1935–6 and 1936–7. In addition, a small subsidiary experiment was carried out with a section of the dairy herd of the National Institute for Research in Dairying during the winter of 1937–8. The planning of this experiment was so different from the large-scale experiments that it is reported separately.

The large-scale experiments were made possible by the voluntary co-operation of a number of farmers, who made available for the experiments the whole or part of their dairy herds. The number of herds co-operating and the counties in which they were situated are shown in Table 1. During the first winter about 500 cows were used and during the second winter over 900.

Table 1. *Number of herds co-operating in field investigation*

	1935–6		1936–7	
County	No. of herds	No. of cows	No. of herds	No. of cows
Berkshire	9	238	7	190
Dorset	1	44	7	248
Hampshire	4	100	6	210
Oxfordshire	—	—	6	172
Somerset	4	112	3	112
Total	18	494	29	932

Uniformity of method was obtained by an initial detailed explanation of the plans to each farmer and by subsequent visits to every farm by a member of the Institute staff at intervals of about 6 weeks.

[1] The protein equivalent of a foodstuff is one-half the sum of the digestible true protein and the digestible crude protein(8).

The rations

The large-scale experiments may be regarded as three separate experiments. In all three, the object was to compare two levels of protein feeding, one supplying approximately the current production standard of 0·6 lb. protein equivalent per 10 lb. milk and the other supplying about two-thirds of this amount.

The usual difficulties associated with the mixing of experimental rations were overcome by the manufacture of the concentrate mixtures into "cubes" or "nuts". In this way two concentrate mixtures similar in appearance and in a form convenient for use were made.

The composition of the cubes used in Exps. 1 and 2 and of those used in Exp. 3 is shown in Table 2. This table shows that 3½ lb. of high-protein cubes supplied about 0·60 lb. protein equivalent and 3½ lb. of low-protein cubes about 0·42 lb. protein equivalent.

Table 2. *Composition of cubes used in Exps.* 1 *and* 2

	High protein %	Low protein %
Ingredients:		
Decorticated ground-nut cake	29·4	12·6
Maize meal	29·4	37·8
Pollards (wheat offal)	29·4	37·8
Molasses	9·8	9·8
Minerals	2·0	2·0
	100·0	100·0
Chemical composition:		
Water	11·5	11·9
Fat	4·6	4·1
Crude protein	21·4	15·6
Soluble carbohydrates	52·0	58·3
Crude fibre	4·8	4·8
Ash	5·7	5·3
	100·0	100·0
Calculated nutrients:		
Starch equivalent	61·85	59·69
Protein equivalent	17·36	11·89
Composition of cubes used in Exp. 3		
Ingredients:		
Decorticated ground-nut cake	16·0	8·0
Soya-bean cake	15·0	7·5
Maize meal	22·5	28·75
Pollards (wheat offal)	12·0	15·0
Rice bran	22·5	28·75
Molasses	10·0	10·0
Minerals	2·0	2·0
	100·0	100·0
Chemical composition:		
Water	14·3	14·4
Fat	6·0	6·5
Crude protein	21·5	16·3
Soluble carbohydrates	48·1	52·9
Crude fibre	4·9	4·8
Ash	5·2	5·1
	100·0	100·0
Calculated nutrients:		
Starch equivalent	65·3	65·3
Protein equivalent	16·9	11·9

Farmers were therefore asked to feed 3½ lb. of cubes for each gallon of milk produced. Food records indicate that on most farms rationing was well controlled and that the recommendation regarding the quantity of cubes was followed. A few herds tended to exceed the recommended quantity of food, but these were almost entirely herds of Channel Island cattle yielding very rich milk. On no farm was there any evidence of a tendency to favour either the high- or low-protein group.

The maintenance rations and hay analyses

It was not possible to ensure that all the cows on the different farms received the same maintenance ration because the supplies of home-grown foods available differed from farm to farm, ranging from hay alone on some farms to kale, mangolds, hay and straw on others. The actual quantities of available foods provided for maintenance were adjusted as far as possible to comply with the accepted standard for maintenance of 6 lb. starch equivalent including 0·6 lb. protein equivalent per 1000 lb. live weight, but, in the absence of full details as to the weights of the cows and the quantity and analysis of each food used, the adjustment could only be approximate. Since hay formed the basis of the maintenance ration on all farms, it was decided to do chemical analyses of at least one sample of hay from each farm during the experiment. The average results for fifty-nine samples of meadow hay, six samples of seeds hay and one sample of lucerne hay are given in Table 3. More detailed analyses of these hays and a discussion thereon has already been published by Davies (7).

Table 3. *Average analyses of hay*

Type of hay	Meadow	Seeds	Lucerne
No. of samples	59	6	1
Moisture	11·69	14·76	14·64
Crude protein	8·32	9·24	14·52
Ether extract	1·80	1·16	0·79
Crude fibre	27·30	26·48	26·55
Mineral matter	6·88	5·81	7·50
Soluble carbohydrates	44·01	42·55	36·00
P_2O_5	0·50	0·50	0·63
CaO	0·87	1·38	1·59

From the food records and hay analyses for each farm, it was possible to estimate the total amount of protein equivalent fed on an average throughout the experimental period. The composition of roughage other than hay was estimated by reference to tables (cf. Wood & Woodman (8)), which are based on the average of a number of analyses. For this reason and because the maintenance rations on some farms were not accurately controlled, the statements as to total quantity must be regarded as estimates.

When the figure for protein equivalent in the maintenance ration of each farm was compared with the standard for the particular breed concerned, there was found to be a general tendency to feed in excess of the accepted

standard. In each experiment the excess protein equivalent of the actual maintenance ration over the recommended standard amounted on an average to about 10 %. In Table 4 the farms are grouped according to the level of protein in the maintenance rations and, as stated in a later paragraph, the possible influence of this factor was considered before conclusions were drawn.

Table 4. *Protein equivalent of maintenance rations*

Number of farms on which the protein equivalent content of the maintenance ratio was:

Exp.	10 % or more below standard	Less than 10 % above or below standard	10–30 % above standard	30 % or more above standard
1	5	6	3	4
2	3	5	9	2
3	3	2	3	2
All 3	11	13	15	8

Plan of experiment

The date of commencement of the experiment varied somewhat from farm to farm, but the investigation can be divided into three periods as follows:

(i) A preliminary control period of 3 weeks or more. During this period in the first experiment the cows were fed the normal farm rations available at the time. In the second and third experiments the concentrates fed to all cows consisted of a mixture of equal weights of "high" and "low" protein cubes.

(ii) An experimental period. In the first experiment this commenced in December 1935 and ended in April 1936. In the second and third experiments it commenced in October or November 1936 and ended in April or May 1937. During this period half the cows on each farm received "high-protein cubes" and the other half "low-protein cubes", but the same maintenance ration was fed to both groups.

(iii) A concluding period, commencing when the cows went to grass and ending 6 weeks later. During this period no differential treatment or rations were allowed to any of the cows; the diet consisted of grass supplemented in some cases with concentrate cubes. It would be possible to regard this period as a final control for the experiment, but it was decided that this was undesirable because the winter experimental rations might have a prolonged residual effect. Results during this grazing period therefore are reported as a separate section and not utilized solely for control purposes.

Cows in all stages of lactation were included in the experiment, but there was a high proportion of recent calvers. This was largely due to the inclusion of herds where only a portion of the cows were available for the experiment, and naturally those cows in the early stages of lactation were chosen. When dividing the cows into groups for high- or low-protein feeding, every effort was made to avoid selection or bias. All available cows on any one farm were grouped in pairs, both animals in each pair being as similar as possible in age, calving dates, milk yield, breed, size, etc. After pairing the cows in this way one cow from each pair was allocated by lot to the high-protein ration, its partner receiving the low-protein ration.

RESULTS

The effects of the high- and low-protein mixtures were judged by the following criteria: fatness of the cow, handling properties of the skin, loss of hair during the winter, yield of milk, and the fat, solids-not-fat and nitrogen content of the milk.

The data collected have been summarized for each experiment, and the results from all three experiments are shown in the appropriate tables.

(*a*) *Effect of level of protein on the condition of the cow*

The live weight of most of the animals was not obtainable, and it was therefore necessary to adopt other methods of assessing condition at the beginning and end of the winter-feeding period. A scale of points was used to assess the physical condition of each cow as shown by (i) fatness, (ii) handling properties of the skin, (iii) loss of hair. In order to obtain uniformity the same person carried out all the assessing in each experiment. The scale of points for each criterion ranged from a maximum of 10 to a minimum of 1. Thus a very fat cow was given 10 marks, a thoroughly emaciated one 1 mark, and intermediate conditions were given marks between 10 and 1. Similarly an ideal, pliable and elastic skin was given 10 marks and a very harsh, leathery skin 1 mark, or a cow completely covered with hair 10 marks, and the reverse 1 mark. This method of assessing condition would not be expected to show up small differences between small groups, but should indicate any consistent difference between large groups such as were used for these experiments.

The average figures concerning the physical condition for the cows in each experiment and for the three experiments together are given in Table 5. These results need little discussion. There was a very slight tendency to lose fat and hair during the winter, but there was no evidence that the low-protein production mixtures had any detrimental effect on the fatness, handling properties or appearance of the cows. The farmers agreed with this conclusion.

Table 5. *Effect of protein level on the condition of the cows*

	Condition or fatness		Handling properties of skin		Loss of hair	
	High-protein cows	Low-protein cows	High-protein cows	Low-protein cows	High-protein cows	Low-protein cows
Experiment 1 (450 cows):						
Mean initial mark	6·17	6·24	6·40	6·47	10·0	10·0
Mean final mark	5·85	5·86	6·69	6·95	9·8	9·8
Mean change during experiment	−0·32	−0·38	+0·29	+0·52	−0·2	−0·2
Experiment 2 (434 cows):						
Mean initial mark	5·12	5·05	4·82	4·87	10·0	10·0
Mean final mark	5·09	5·07	4·94	4·88	9·6	9·7
Mean change during experiment	−0·03	+0·02	+0·12	+0·01	−0·4	−0·3
Experiment 3 (212 cows):						
Mean initial mark	4·85	5·04	5·02	4·89	10·0	10·0
Mean final mark	4·78	4·95	4·64	4·54	9·7	9·8
Mean change during experiment	−0·07	−0·09	−0·38	−0·35	−0·3	−0·2
All experiments (1096 cows):						
Mean initial mark	5·50	5·53	5·51	5·53	10·0	10·0
Mean final mark	5·36	5·37	5·60	5·67	9·7	9·7
Mean change during experiment	−0·14	−0·16	+0·09	+0·14	−0·3	−0·3

(*b*) *Effect of level of protein on the yield of quality and milk*

(i) *Milk yield during the winter.* Throughout the winter the total number of cows in milk in any group varied owing to drying off and calving. Also those in milk were in various stages of lactation. The results have been classified and studied in a variety of ways and Table 6 presents one of these classifications, viz. the average weekly yields of all cows in milk in any particular group as long as they complied with the following conditions:

(*a*) The yield of a cow was included only when the yield of its partner was available.

(*b*) Yields from any pair of cows differing in calving dates by more than three months were excluded.

(*c*) Yields were included only from the first complete week of lactation till the cows had been in calf six months or had become dry.

It will be realized that the records of most of the cows calving in the autumn were included throughout the winter, whereas the records of the winter and spring calvers were excluded for a time but were again included as they and their respective partners calved.

In Table 6 the average weekly milk yields of the cows receiving the high- and the low-protein mixtures are shown for each of the three experiments. In all three slight changes in yields occurred from week to week, due partly to normal lactational changes in yield and partly to changes in the numbers of pairs of experimental cows owing to calving, drying off, etc. The milk-producing value of the two rations is indicated by comparison between the high- and the low-protein cows and in this respect there is a remarkable similarity. Thus, in Exps. 1 and 3 the high-protein cows gave slightly more milk than their low-protein counterparts during the initial control period and also during the experimental period. In Exp. 2 the low-protein cows gave higher yields during the initial control and the experimental periods. When the results of the three experiments were bulked together the difference between the two levels of protein was negligible, the mean weekly yields during the preliminary control period being for high-protein cows 179·3 lb. and for low-protein cows 178·6 lb., while during the experimental period both groups averaged 172·1 lb. weekly.

A second and alternative classification was made by dividing the experimental period into sections of 10 weeks and including only pairs which remained in milk throughout these shorter periods; by this method it was possible to obtain a series of milk-yield curves which included most of the data without the irregularities of a changing population. For the sake of brevity the tables and curves for this second analysis of milk-yield data are not presented here. It is, however, possible to state categorically, that there was no evidence of any difference between the mean yield of the high- and low-protein cows.

A third classification of the milk-yield data was designed to show the effects, if any, of the level of protein in the maintenance rations. No consistent

Table 6. *Mean weekly milk yield during winter feeding period*

	Exp. 1			Exp. 2			Exp. 3			All 3 experiments together		
	No. of pairs	High protein lb.	Low protein lb.	No. of pairs	High protein lb.	Low protein lb.	No. of pairs	High protein lb.	Low protein lb.	No. of pairs	High protein lb.	Low protein lb.
Preliminary control period:												
1st week	142	165·9	162·9	188	187·2	188·7	86	194·4	195·3	416	181·4	181·3
2nd week	140	161·7	157·9	194	184·0	185·9	89	196·2	189·9	423	179·2	177·5
3rd week	145	162·2	160·3	199	179·8	181·7	93	195·5	193·5	437	177·3	177·1
Mean of control period		163·3	160·4		183·7	185·4		195·4	192·9		179·3	178·6
Experimental period:												
1st week	148	159·4	157·5	193	181·4	180·6	94	190·5	188·2	435	175·9	174·4
2nd week	151	154·3	153·1	199	177·5	175·7	95	201·1	193·0	445	174·7	171·7
3rd week	151	153·5	154·2	203	176·3	174·8	95	196·9	189·6	449	173·0	171·0
4th week	146	154·0	156·0	196	172·5	173·1	94	195·1	192·5	436	171·2	171·6
5th week	141	154·4	157·1	196	171·9	169·7	96	194·5	190·0	433	171·2	170·1
6th week	138	152·4	158·1	194	171·1	167·5	95	189·7	190·1	427	169·2	169·5
7th week	138	153·3	156·0	192	168·7	169·8	96	184·3	182·2	426	167·3	168·1
8th week	139	155·0	156·6	197	170·2	171·8	94	191·2	185·6	430	169·9	169·9
9th week	140	157·2	159·8	195	171·9	174·6	96	191·7	196·0	431	171·5	174·6
10th week	139	156·4	156·1	199	174·0	176·3	95	193·0	188·8	433	172·5	172·6
11th week	133	159·6	156·8	193	171·1	173·8	95	193·5	185·5	421	172·5	171·1
12th week	136	157·8	154·5	193	167·1	171·8	95	192·4	188·1	424	169·8	169·9
13th week	139	154·8	152·1	194	172·2	175·3	95	190·2	185·9	428	170·5	170·1
14th week	138	158·6	156·2	194	170·2	174·4	97	187·2	180·9	429	170·3	170·0
15th week	139	159·1	156·4	191	172·7	177·6	98	186·8	184·8	428	171·5	172·3
16th week	139	159·2	158·8	181	173·0	176·7	96	187·2	184·4	416	171·7	172·5
17th week	136	160·7	161·0	177	170·3	176·1	98	190·4	183·4	411	171·9	172·9
18th week	139	161·8	162·2	173	167·5	175·1	98	186·1	196·5	410	170·0	171·1
19th week	140	163·8	164·1	174	163·5	169·8	95	182·9	177·3	409	168·1	169·6
Mean of 19 weeks experimental period		157·1	157·2		171·4	173·6		190·4	187·1		171·2	171·2

differences were found, however, between the results from farms where the maintenance rations were high, medium or low in protein. A fourth classification indicated that no measurable difference could be observed between the response to the two diets between cows of high and low producing capacity.

Throughout the three experiments during the winter feeding period, therefore, there was no evidence, in any of the analyses of the data, of a consistent difference between the milk yield of the cows on the high- and on the low-protein rations.

(ii) *Milk yield during the change to spring grazing.* Under normal English conditions the change from winter to summer rations is somewhat sudden, and the summer diet of cows consists almost entirely of grass supplemented, in some cases, with a small quantity of concentrates. The farms taking part in the experiments followed their normal practice, but when winter feeding was deemed to be finished only one concentrate ration was fed to all cows. In

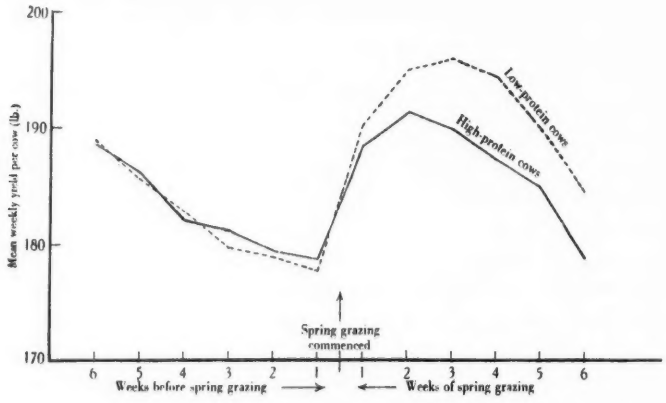

Fig. 1. Milk yield of 194 pairs of cows during the change from winter rations to grazing. (Exps. 1 and 2 combined.)

Exp. 1 this concentrate consisted of the low-protein cubes and in Exps. 2 and 3 it consisted of a mixture of equal weights of high- and low-protein cubes. It was not easy to decide the exact date of change from winter to summer feeding on all farms; in some cases the date was clearly marked, but in others where the change was more gradual it was necessary to fix a date after which cows were assumed to have an appreciable quantity of grass.

Milk records were collected for the first 6 weeks of grazing and these have been summarized in Table 7. In the preparation of this table the only records used were those of pairs of cows which remained in milk throughout the 12 weeks' period including 6 weeks before to 6 weeks after the spring change. The results for each separate experiment, for Exps. 1 and 2 combined and for all three experiments combined are shown in Table 7. The results of Exps. 1 and 2 combined are also shown graphically in Fig. 1.

Table 7. *Mean weekly yield before and after commencement of spring grazing*

	Exp. 1			Exp. 2			Exps. 1 and 2 combined			Exp. 3			Exps. 1, 2 and 3 combined		
	No. of pairs included	High-protein group lb.	Low-protein group lb.	No. of pairs included	High-protein group lb.	Low-protein group lb.	No. of pairs included	High-protein group lb.	Low-protein group lb.	No. of pairs included	High-protein group lb.	Low-protein group lb.	No. of pairs included	High-protein group lb.	Low-protein group lb.
6th week before grazing	89	187·6	183·2	105	189·6	193·8	194	188·6	188·9	58	210·1	204·3	252	193·6	192·5
5th week before grazing	89	184·0	180·0	105	187·8	190·3	194	186·2	185·6	58	201·5	197·2	252	189·8	188·2
4th week before grazing	89	179·3	177·9	105	184·5	186·9	194	182·1	182·8	58	202·1	196·9	252	186·7	186·0
3rd week before grazing	89	177·7	174·8	105	184·1	183·9	194	181·2	179·7	58	203·9	195·1	252	186·4	183·3
2nd week before grazing	89	174·2	172·3	105	184·0	184·6	194	179·5	178·9	58	202·8	194·0	252	184·8	182·4
1st week before grazing	89	170·4	167·8	105	185·9	186·4	194	178·8	177·8	58	199·9	191·9	252	183·7	181·1
1st week of grazing	89	185·7	188·0	105	190·4	192·1	194	188·3	190·2	58	212·8	204·9	252	193·9	193·6
2nd week of grazing	89	187·5	190·5	105	194·4	198·7	194	191·2	194·9	58	215·3	209·0	252	196·8	198·2
3rd week of grazing	89	183·1	188·5	105	195·7	202·1	194	189·9	195·8	58	218·3	206·9	252	196·5	198·4
4th week of grazing	89	179·3	185·7	105	193·9	201·6	194	187·2	194·3	58	214·8	205·5	252	193·6	196·9
5th week of grazing	89	174·6	180·8	105	193·6	197·7	194	184·9	190·0	58	211·0	206·4	252	190·9	193·7
6th week of grazing	89	167·6	174·6	105	188·5	192·8	194	178·9	184·5	58	207·1	202·6	252	185·4	188·6

As might be expected, there was in each experiment a rise in average weekly milk production at the commencement of spring grazing. A point of much greater importance, however, noticeable in Exps. 1 and 2 but not in Exp. 3, was the difference between the increase of the high- compared with the low-protein fed cows. This difference was found to be statistically significant; for example, comparing the milk yield 1 week prior to spring grazing with the yields 3 weeks later, the low-protein cows increased their production about 7 lb. per cow per week more than the corresponding high-protein cows. Such a difference would only occur by chance once in 2500 similar trials. This finding is capable of more than one explanation and will therefore be dealt with later in the discussion.

(iii) *Fat, solids-not-fat and nitrogen content of the milk.* Samples of the milk yielded in one complete day were taken from each cow at intervals of about 6 weeks throughout the winter-feeding experimental period. In the second and third experiments control samples were taken from all cows in-milk before the high- and low-protein feeding commenced and in all three experiments samples were taken during the spring grazing period after the experimental feeding had ceased.

The samples were sent to the National Institute for Research in Dairying and the following tests made: (*a*) Fat percentage of each sample by the Gerber method. (*b*) Solids-not-fat percentage of each sample. In the first experiment this was done by the gravimetric method (Golding's modification) and in the second and third experiments by calculation from the density and fat percentage. In Exp. 1 the protein nitrogen was determined on composite samples prepared from the milk of the cows on each farm receiving the high- and low-protein rations respectively. The method used was precipitation by trichloracetic acid and Kjeldahl determinations on the precipitate.

The results of the fat tests are summarized in Table 8. In Exps. 1 and 2

Table 8. *Percentage fat content of milk*

	Test during preliminary period	Tests during winter feeding period			Last test during winter feeding period	First test during grazing period
		1st	2nd	3rd		
Exp. 1:						
No. of pairs	—	137	128	133	140	123
High-protein cows	—	3·89	3·69	3·74	3·55	3·42
Low-protein cows	—	3·93	3·70	3·69	3·59	3·47
Exp. 2:						
No. of pairs	179	182	193	160	163	135
High-protein cows	3·75	3·88	3·90	3·80	3·85	3·48
Low-protein cows	3·69	3·93	3·88	3·86	3·92	3·56
Exp. 3:						
No. of pairs	81	90	94	97	91	66
High-protein cows	3·77	3·89	3·68	3·69	3·68	3·49
Low-protein cows	3·95	3·80	3·53	3·47	3·49	3·45
Exps. 1, 2 and 3, combined:						
No. of pairs	—	409	415	390	394	324
High-protein cows	—	3·89	3·78	3·75	3·70	3·46
Low-protein cows	—	3·90	3·75	3·71	3·70	3·51

the high- and low-protein cows showed almost identical results, the general level of fat percentage in both being reasonably constant throughout the winter with the usual seasonal decrease in spring. In Exp. 3 there was a decrease in fat percentage during the course of the winter, and this was more pronounced in the low- than in the high-protein cows. Neither the changes during the winter nor the differences in behaviour of the two groups were statistically significant and therefore may possibly be due to accidental causes. If, however, these changes were in fact due to nutritional causes, it is difficult to ascribe them to the levels of protein fed: it is more likely that they would be due to the effect of one of the ingredients of concentrate cubes used in Exp. 3. The only two ingredients of the mixtures used in Exp. 3 which were not used in Exps. 1 and 2 were soya-bean meal and rice bran, and of these two foods greater suspicion is cast on the rice bran because it constituted a larger proportion of the low-protein mixture.

The results of the solids-not-fat tests are summarized in Table 9. They are so consistent in all groups that little comment is necessary, and there is no

Table 9. *Percentage solids-not-fat content of milk*

	Test during preliminary control period	Tests during winter feeding period			Last test during winter feeding period	First test during grazing period
		1st	2nd	3rd		
Exp. 1:						
No. of pairs	—	137	128	133	140	123
High-protein cows	—	8·80	8·76	8·75	8·69	8·80
Low-protein cows	—	8·84	8·76	8·77	8·74	8·89
Exp. 2:						
No. of pairs	179	182	193	160	163	135
High-protein cows	8·99	8·77	8·75	8·73	8·65	8·83
Low-protein cows	9·01	8·77	8·70	8·71	8·61	8·80
Exp. 3:						
No. of pairs	81	90	94	97	91	66
High-protein cows	8·71	8·68	8·60	8·61	8·65	8·73
Low-protein cows	8·69	8·64	8·60	8·59	8·59	8·72
Exps. 1, 2, and 3 combined:						
No. of pairs	—	409	415	390	394	324
High-protein cows	—	8·76	8·72	8·71	8·67	8·80
Low-protein cows	—	8·76	8·70	8·70	8·65	8·82

evidence that the level of dietary protein affected the results. All groups showed a slight increase in the solids-not-fat percentage after grazing commenced; this is a common seasonal change, but it is not known whether it is due to nutritional causes or to some other seasonal factor. It may be noted that Foot & Shattock (9) have already studied this data with reference to the relationship between cows yielding milk low in solids-not-fat and those infected with mastitis. Further reference to this subject is therefore unnecessary.

The protein-nitrogen content of the milk was almost identical in the two dietary groups. During the experimental feeding period the mean values were

for high-protein cows 0·470 % nitrogen and for low-protein cows 0·472 % nitrogen, while during the grazing period both values were higher, viz. for high-protein cows 0·491 % and for low-protein cows 0·492 %.

Exp. 4. In addition to the three field experiments described above, a fourth experiment was carried out at the National Institute for Research in Dairying during the winter of 1937–8. Although this included only a small number of cows, the experimental conditions permitted much more careful control of rations, live weights and results.

The object of this experiment was to throw further light on certain results obtained in the large-scale experiments and to provide a direct comparison between the two types of concentrate mixtures previously used, one containing ingredients from more varied sources than the other.

The production rations in Exp. 4 were mixed on the farm and were, therefore, not in the form of cubes. Three rations were used, two corresponding to the high- and low-protein cubes of the first two field experiments, and one corresponding to the low-protein cubes of the third experiment. The mixtures, called respectively H 1, L 1 and L 3, were made from the same foods as the corresponding cubes except that the molasses was omitted. Mixture L 3 therefore differed from the other two in containing rice meal and soya-bean meal. The proportions of the various foods were adjusted to give a starch equivalent and protein equivalent similar to those of the corresponding cubes.

Five blocks of cows were used, one member of each block being chosen by lot for each concentrate mixture. Thus there were five cows on each treatment. A preliminary control period of 3 weeks was followed by an experimental period of 13 weeks, and this again by a concluding or grazing period of 5 weeks. During the preliminary control and concluding periods all fifteen cows were similarly fed on a concentrate mixture which was a composite of mixtures H 1, L 1 and L 3.

The maintenance ration, which consisted of hay, mangolds, silage and a little flaked maize, was calculated for each cow on the basis of its actual live weight. The production ration for each cow was periodically adjusted to yield.

It is not proposed to give the results of this experiment in detail. The important data are summarized in Table 10, and interpretation of the figures has been simplified by expressing the results of the experimental and concluding periods as a percentage of the preliminary control period. The number of cows used for the experiment was not sufficient to enable definite conclusions to be drawn, but the experiment provides some confirmation of certain conclusions from the field experiments. Thus the milk yield response to rations H 1 and L 1 was similar to that observed in Exps. 1 and 2, i.e. almost identical yields during the experimental period, while the low-protein group, L 1, gave higher yields when grazing commenced. Also the cows receiving ration L 3 (including soya-bean and rice bran) showed the greatest fall in the milk fat

percentage during the experimental period. Apart from this the results did not suggest any important differences between the three rations in effect on milk yield, milk composition or live weight.

Table 10. *Results of Exp.* 4

The figures enclosed in brackets ( ) represent the values of each period expressed as a percentage of the preliminary control period

	Production ration*	Preliminary control period 3 weeks 9–30 Jan.	Experimental period 13 weeks 31 Jan. to 30 Apr.	Concluding or grazing period 5 weeks 1 May to 4 June
Milk yield, weekly average per cow, lb.	H 1	198·2 (100)	178·7 (90·2)	169·0 (85·3)
	L 1	206·9 (100)	186·3 (90·0)	185·7 (89·8)
	L 3	198·9 (100)	180·7 (90·8)	172·3 (86·6)
Milk fat percentage	H 1	3·72 (100)	3·68 (98·9)	3·84 (103·2)
	L 1	3·67 (100)	3·42 (93·2)	3·32 (90·5)
	L 3	3·71 (100)	3·38 (91·1)	3·37 (90·8)
Solids-not-fat percentage	H 1	9·05 (100)	9·02 (99·7)	9·35 (103·3)
	L 1	8·81 (100)	8·87 (100·7)	9·02 (102·4)
	L 3	9·08 (100)	8·99 (99·0)	9·18 (101·1)
Mean live weight at end of period, lb.	H 1	1154 (100)	1161 (100·6)	1124 (106·1)
	L 1	1140 (100)	1166 (102·3)	1195 (104·8)
	L 3	1159 (100)	1172 (101·1)	1234 (106·5)

* H 1 = a concentrate mixture based on the composition of the high-protein cubes used in Exps. 1 and 2.

L 1 = a concentrate mixture based on the composition of the low-protein cubes used in Exps. 1 and 2.

L 3 = a concentrate mixture based on the composition of the low-protein cubes used in Exp. 3.

Discussion

The primary object of these experiments is believed to have been attained in that they showed that a production standard of 0·6 lb. protein equivalent per 10 lb. milk is unnecessarily high under existing practical farming conditions in the south of England. Certain secondary results were noted during the course of the experiment, and it is of interest to consider whether these were due solely to the protein levels of the diet. While the production rations contained different quantities of protein, it is important to realize that such differences were, of necessity, accompanied by slight differences in the non-protein fraction. In the interpretation of the results, therefore, it is necessary to consider the effect of the non-protein as well as the protein fraction of the ration.

An unexpected and subsidiary result was the greater response in milk yield to spring grazing shown by the low- as opposed to the high-protein fed cows in the three Exps. 1, 2 and 4. One possible explanation of this result is that a greater increase in yield on grass indicates that the winter ration was less efficient for milk production, but a strong objection to this explanation is that the yields during the winter of the low-protein fed cows did not show the slightest indication of inferiority to those receiving the high-protein ration.

A more satisfactory explanation is based on the types of nutrients stored in the bodies of animals when the protein intake is at different levels. Thus Johnson *et al.* (10) showed that rats on a higher protein diet stored more water, protein and ash than their paired mates on a low-protein diet, which stored more fat. From this it seems possible that the condition of the flesh or the type of nutrients stored by the low-protein fed cows may have "fitted" them better for response to the stimulus of young grass.

Neither of these explanations is confirmed by Exp. 3, but this experiment was less satisfactory because many of the cows suffered from mild digestive disturbances. The results therefore do not have the same reliability as those obtained in Exps. 1 and 2. However, the main purpose of Exp. 3 was to note the effect, if any, of protein from more varied sources on the results during the winter and all that need be stated is that the high- and the low-protein ration proved so similar in milk production value that it is unnecessary to modify the main conclusion noted in the opening sentence of this discussion.

Summary

1. In a large-scale experiment, using about 500 cows, half the cows were fed a production ration consisting of decorticated ground-nut cake, maize meal, wheat offals and molasses supplying about 0·6 lb. protein equivalent per 10 lb. milk produced, and the remaining half were fed a production ration containing the same constituent foods in different proportions supplying a similar starch equivalent, but only two-thirds the amount of protein. No measurable difference was observed in the fatness, handling properties of the skin, hair on the body and percentage of fat and solids-not-fat in the milk.

The milk yields showed no difference during the winter feeding period of about 20 weeks, but during the succeeding period when all the cows were allowed ample grass the milk yield response of the cows previously given the low-protein ration was significantly greater than that of the corresponding cows previously given the high-protein ration.

The experiment was repeated the following winter, using about 600 cows, and confirmatory results were obtained.

2. Another experiment similarly planned, using 300 cows, compared high- with low-protein production rations consisting of the constituents noted above together with soya-bean meal and rice bran. No significant differences were observed between the effect of the two rations on milk yield or composition, fatness of the cows, etc. There was, however, an indication (not statistically significant) that the low-protein ration which contained a relatively high proportion of rice bran depressed the milkfat percentage.

3. A small but more completely controlled experiment confirmed the findings of the large-scale experiments.

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A list in detail of all those who helped to make these experiments a success would be far too long to give here. To the co-operating farmers who, in addition to providing experimental cows, gave courteous advice and hospitality; the farms staffs who, in general, took an intelligent interest in the work; the County Agricultural staffs who helped in finding suitable farms; Messrs Lever Bros. who made the rations into convenient cubes; the Milk Recording Society staffs, who took the milk samples for chemical analysis; the Chemical Department of the National Institute for Research in Dairying who carried out all the chemical analytical work; H. A. L. Flatt and V. I. Lovesay who, as post-graduate students, were chiefly responsible for carefully conducting Experiment 4; and to others who helped in various ways we wish to tender our grateful thanks.

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257. THE INFLUENCE OF VARIOUS FACTORS ON THE FERMENTATION END-PRODUCTS OF THE HETEROFERMENTATIVE LACTOBACILLI

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In a previous paper (1) the influence of temperature, *p*H, oxygen tension and the presence of "growth factors" (addition of yeast autolysate) on the ratio of end-products formed by the heterofermentative streptococci was discussed. Using identical experimental conditions, the investigation has been extended to the analogous heterofermentative lactobacilli, some homofermentative types being again included for comparison. In addition, an attempt was made to determine whether excess chalk in the medium had any influence other than that of neutralizing developed acidity, and for this purpose experiments were made with four organisms in which the chalk was replaced by talc.

The group of organisms under discussion has received little intensive study except from systematists, Orla-Jensen (2), Henneberg (3) and Pederson (4) having made the most outstanding contributions to our knowledge of the lactobacilli. Peterson *et al.* (5) have investigated the influence of carbon source on the proportion of by-products formed, particularly in the case of *L. pentoaceticus*. The thirteen strains studied during the present investigation are listed in Table 1. They were selected from the stock cultures maintained in this Institute by Dr J. G. Davis, whose classification (6, 7) of these organisms has been used in this paper.

Methods

With slight modifications the methods used for the streptococci were found satisfactory. The greatest difficulty lay in the irregular nature of the growth when small inocula of these fastidious organisms were used. In many cases the inoculum died out time after time and it was finally found necessary to add about 2 % of dextrose to all media. The sugar was added as an autoclaved 50 % solution to the medium which had already been sterilized in bulk by intermittent steaming, as previously described.(1) Unfortunately, with free dextrose in the medium no simple analytical procedure could determine to what extent residual lactose had become hydrolysed, as preferential use of one or other of the sugars was possible. Accordingly, this aspect of the behaviour of lactobacilli in milk had to be neglected.

In those experiments where talc was substituted for chalk, 10 g. of ignited talc were added to the medium in each flask as required.

Table 1. *Sources of strains and classification*

Name	Code name	Name under which received	Source
L. plantarum	5 O.J.	*Sbm. plantarum*	S. Orla-Jensen (Copenhagen)
L. casei	W. 5	Unnamed	I. R. Sherwood (Palmerston North, N.Z.)
L. acidophilus	1724	*L. acidophilus*	National Collection of Type Cultures, Lister Institute, London
L. ? (intermediate between homofermentative and heterofermentative types)	W. 4	Unnamed	I. R. Sherwood (Palmerston North, N.Z.)
L. bifidus	2797	*L. bifidus*	National Collection of Type Cultures, Lister Institute, London
L. bifidus	4034	*L. acidophil-aerogenes*	National Collection of Type Cultures, Lister Institute, London
L. fermenti	R 1	*Bbm. longum*	W. Ritter, Berne, Switzerland
L. pentoaceticus (?)	W. 1	Unnamed	I. R. Sherwood (Palmerston North, N.Z.)
L. pentoaceticus (?)	W. 2	Unnamed	I. R. Sherwood (Palmerston North, N.Z.)
L. buchneri Pederson(4) and Henneberg(3)	P. 3	*L. mannitopoeus*	C. S. Pederson, Geneva, U.S.A.
L. pentoaceticus	L. 28	*L. brassicae fermentatae*	C. S. Pederson, Geneva, U.S.A.
L. pentoaceticus	947	*L. pentoaceticus*	National Collection of Type Cultures, Lister Institute, London
L. pastorianus	Shim.	*L. pastorianus*	J. L. Shimwell, London

DISCUSSION

The main analytical results are given in Table 2, which includes the experiments using talc in place of chalk. The sugar utilizations (as total hexose anhydride) and total production of lactic and acetic acids and alcohol are stated as millimols per 100 ml. of medium, and the calculated ratios of end-products formed to (*a*) sugar disappearing from the medium and (*b*) lactic acid formed, are also given. As in previous work with streptococci(1) the carbon recovery figures (Table 2) are variable and, since the sugar determinations are subject to errors which cannot be estimated, the ratios of acetic acid and alcohol to both the sugar disappearing from the medium and to the lactic acid produced have been calculated. Whichever ratio is considered, however, the conclusions are identical.

Generally speaking, the results obtained with the lactobacilli are much less satisfactory than those obtained with the streptococci. The differences due to the four factors studied are more variable and frequently even contradictory. Whether it is that when working with such delicate and unstable organisms the isolation of a single variable is a matter of increased difficulty, or whether it is that the *Lactobacillus* genus is less homogeneous than the corresponding streptococcal group, the fact remains that with similar experimental procedures the response with the rod forms is less marked than with the streptococci.

Total growth

With the heterofermentative types little or no growth (as measured by sugar utilization) occurred in the plain milk media but with the homofermentative types (*L. plantarum*, *L. casei*, *L. acidophilus*) the addition of yeast did not greatly affect the total growth.

Lactic acid

The total production of lactic acid is increased by the presence of chalk and growth factors (heterofermentative types), but is not affected by temperature or oxygen tension. The ratio of lactic acid produced to sugar disappearing from the medium is unaffected by temperature, oxygen tension, or the presence of yeast in the medium, but is increased by chalk.

Acetic acid

The total amount of acetic acid produced is greater in the presence of chalk and "growth factors" and under aerobic conditions. Temperature, however, has little effect although with the streptococci the lower temperatures considerably increase acetic acid production.

Considering the ratios of acetic acid produced to sugar utilized and lactic acid formed it is seen that relatively less acetic acid is formed in the presence of chalk and under anaerobic conditions, and there is a tendency for less in the presence of yeast and less at the higher temperature. Orla-Jensen (2) found that chalk did not affect the ratio of acetic acid formed to sugar utilized and that comparatively more acetic acid was produced at the lower temperature, but the results reported here are consistent with the previous findings for streptococci (1).

Alcohol

The total production of alcohol is increased by anaerobiosis, and also by the presence of chalk and yeast in the medium, but temperature has little effect. The ratios of alcohol formed to sugar utilized and lactic acid produced are greatly increased by anaerobic conditions, and to a less extent by the presence of chalk; yeast and temperature, however, have little influence.

Comparison of findings for streptococci and lactobacilli

In Table 3 the effects of the four factors investigated on the total production of lactic and acetic acids and alcohol, and the ratios of these end-products to the sugar utilized, are summarized for both streptococci (1) and lactobacilli. In general the findings for the two groups of bacteria are identical, but the influence of temperature which is evident with the streptococci is almost absent in the rod forms. Yeast autolysate also has less influence with the lactobacilli.

Table 3. *Summary of findings with heterofermentative streptococci and lactobacilli. Influence of four factors investigated on the total production of end-products and ratios of end-products to sugar utilized*

			Restricted oxygen supply	Presence of chalk	Presence of yeast autolysate	Decrease in temperature
Lactic acid	Streptococci	Total	Increased	Increased	Increased	Increased
		Ratio	Increased	Increased	Variable	Variable
	Lactobacilli	Total	Variable	Increased	Increased	Variable
		Ratio	Variable	Increased	Variable	Decreased
Acetic acid	Streptococci	Total	Decreased	Increased	Increased	Increased
		Ratio	Decreased	Decreased	Decreased	Decreased
	Lactobacilli	Total	Decreased	Increased	Increased	No effect
		Ratio	Decreased	Decreased	Slightly decreased	Slightly increased
Alcohol	Streptococci	Total	Increased	Increased	Increased	Increased
		Ratio	Increased	Increased	Increased	Increased
	Lactobacilli	Total	Increased	Considerably increased	Increased	Variable
		Ratio	Increased	Slightly increased	Variable	Variable

Ratio of by-products to total end-products

The by-products estimated were acetic acid and alcohol (carbon dioxide omitted) and the percentages that these formed of the total end-products (by-products + lactic acid) are given in Table 4. With the homofermentative types, 10 % or less of the total end-products are alcohol and acetic acid, but with the heterofermentative types the proportion increases to 40–50 %. *L. bifidus* (strain 4034) is exceptional in that 50–60 % of the total end-products consists of acetic acid and alcohol.

The effect of adding talc to the medium

In the experiments with the four organisms P. 3, L. 28, 947, and Shim., the usual series of cultures containing chalk was omitted and replaced by cultures containing about the same volume of finely ground talc. These cultures were treated in exactly the same way as the usual chalk cultures. The results obtained, using talc, are given at the end of Table 2.

The presence of talc did not affect the extent of total growth as measured by sugar utilization, but the ratio of lactic acid formed to sugar used and also the ratios of alcohol produced to sugar used and lactic acid formed are changed. It will be seen that talc confers anaerobic properties even upon the "aerobic" cultures, i.e. oxygen has less effect than when talc is absent. The "anaerobic" cultures which had a layer of liquid paraffin over the medium to minimize the entry of oxygen also show an accentuation of anaerobic characters, i.e. a higher proportion of the sugar is degraded to lactic acid and alcohol. That this should occur in the "anaerobic" as well as the aerobic cultures is explicable when it is remembered that the degree of anaerobiosis attained under liquid paraffin is not high. The rate of exchange of free oxygen across a 2 cm. layer of paraffin is quite appreciable (8).

Table 4. *Ratio of by-products to total end-products* (%)

$$\frac{(\text{Acetic acid} + \text{alcohol}) \times 100}{(\text{Lactic acid} + \text{acetic acid} + \text{alcohol})} \text{ weight basis}$$

Strain		Ae.	Ae.C.	An.	An.C.
L. plantarum: 5 O.J.	S. 37	8	6	10	9
	S. 19	12	4	6	6
	Y. 37	9	4	9	3
	Y. 19	15	3	7	3
L. casei: W. 5	S. 37	4	N.D.	6	N.D.
	S. 19	5	N.D.	3	N.D.
	Y. 37	3	N.D.	8	N.D.
	Y. 19	5	N.D.	4	N.D.
L. acidophilus: 1724	S. 37	6	5	6	4
	S. 25	10	N.D.	6	5
	Y. 37	8	6	7	3
	Y. 25	7	7	10	7
L. ? W. 4	S. 37	8	7	2	6
	S. 19	N.D.	N.D.	N.D.	N.D.
	Y. 37	8	6	10	5
	Y. 19	N.D.	N.D.	N.D.	N.D.
L. bifidus: 2797	S. 32	40	46	43	38
	S. 19	36	N.D.	—	50
	Y. 32	41	37	47	41
	Y. 19	47	37	47	40
4034	S. 37	44	70	76	71
	S. 19	45	57	55	66
	Y. 37	50	52	57	52
	Y. 19	50	58	52	54
L. fermenti: R. 1	S. 37	40	40	42	44
	S. 19	—	N.D.	45	54
	Y. 37	35	33	38	33
	Y. 19	45	41	44	42
L. pentoaceticus: W. 1	S. 37	37	N.D.	31	N.D.
	S. 19	46	N.D.	—	N.D.
	Y. 37	42	N.D.	35	N.D.
	Y. 19	47	N.D.	55	N.D.
W. 2	S. 37	38	36	36	33
	S. 19	N.D.	N.D.	N.D.	N.D.
	Y. 37	37	33	37	29
	Y. 19	N.D.	N.D.	N.D.	N.D.

		Ae.	Ae.T.	An.	An.T.
L. buchneri: P. 3	S. 32, S. 19	No significant growth			
	Y. 32	39	37	42	39
	Y. 19	38	40	43	35
L. pentoaceticus: L. 28	S. 37, S. 19	No significant growth			
	Y. 37	48	48	49	53
	Y. 19	44	40	49	47
947	S. 37, S. 19	No significant growth			
	Y. 37	43	56	61	57
	Y. 19	54	55	54	60
L. pastorianus: Shim.	S. 32, S. 19	No significant growth			
	Y. 32	48	51	52	50
	Y. 19	44	47	41	50

Ae. = aerobic. An. = anaerobic. C. = chalk. T. = talc.
S. = separated milk. Y. = yeast separated milk. 19, 25, 32, 37 = ° C. N.D. = not done.

As the number of experiments using talc was limited, *t*-tests were applied to the results. Considering the ratio of lactic acid produced to sugar utilized, the difference due to the presence of talc is not significant when all the results are considered together, but it may be noticed that the three cases in which a *decrease* in the ratio occurs are experiments carried out under anaerobic conditions at 19°. Excluding this series of four experiments, the remaining figures show a highly significant increase in the proportion of lactic acid formed from sugar. (The level of significance used throughout is $P < 0{\cdot}01$.)

The ratio of alcohol formed to sugar utilized is also increased in the presence of talc. A *t*-test applied to the series of results for all four organisms shows a significant difference due to talc, but the level of significance is $P = 0{\cdot}03$. Inspection of the figures, however, shows that with P. 3 and L. 28 the differences are not consistent. Applying *t*-tests to the results for the pairs of organisms P. 3 and L. 28 and 947 and Shim. separately, it was shown that the differences found with P. 3 and L. 28 are not significant, while the differences shown by 947 and Shim. are highly significant.

In the same manner as freshly minced tissue favours the growth of anaerobes, the effect of talc in accentuating the anaerobic character of the fermentation is no doubt related to the increased internal surface of the medium (cf. Breden & Buswell (9), who used shredded asbestos in liquid medium for the propagation of methane-producing organisms). It appears that surface area due to colloidal material does not affect the nature of the fermentation, since the internal surface of milk colloids is vast compared with the surface of the talc added in these experiments. Since both the talc and chalk used were of comparable particle size, it is interesting to speculate on the mechanism by which chalk added to the medium increases the proportion of lactic acid and alcohol formed from the sugar. It was originally intended that the chalk should prevent a rapid decrease in the *p*H of the medium, and in previous experiments with streptococci (1) the effect of chalk was assumed to be connected with a higher *p*H. But now that it has been shown that talc has just as strong an influence on the ratios of by-products formed, it seems that the effect of chalk may be entirely or partly due to the increased effective surface. Until further experiments are carried out in which the *p*H is controlled accurately by addition of strong alkali as well as by chalk, this question must remain open.

*Final p*H *values*

These values are given in Table 5 from which it may be seen that the lowest values occur in the experiments with yeast separated milk incubated at the higher temperature.

Orla-Jensen (2) found that the lowest *p*H values occurred when the incubation temperature was below the optimum, and previous results with streptococci (1) were in agreement with his findings, which were general for the lactic acid bacteria. It is possible that in the present experiments an incubation time of 8 weeks at 19° was too short to allow exhaustive growth.

Table 5. *Final* pH *values*

Strain		Control	Ae.	Ae.C.	An.	An.C.
L. plantarum: 5 O.J.	S. 37	6·37	4·50	6·52	4·73	4·96
	S. 19	6·35	4·73	5·66	4·06	5·29
	Y. 37	6·17	4·02	4·16	4·01	4·93
	Y. 19	6·29	4·13	4·29	3·91	4·22
L. casei: W. 5	S. 37	6·30	3·76	—	3·76	—
	S. 19	6·40	3·82	—	3·63	—
	Y. 37	6·30	3·70	—	3·75	—
	Y. 19	6·30	3·58	—	3·58	—
L. acidophilus: 1724	S. 37	6·20	3·52	4·21	3·41	4·88
	S. 25	6·20	4·18	N.D.	3·78	4·97
	Y. 37	6·22	3·67	5·48	3·60	4·07
	Y. 25	6·14	4·01	5·45	4·13	5·00
L. bifidus: 2797	S. 32	6·28	5·30	6·89	5·47	5·77
	S. 19	N.D.	N.D.	N.D.	N.D.	N.D.
	Y. 32	6·15	3·57	5·20	3·55	5·57
	Y. 19	6·20	3·68	5·86	3·68	5·28
4034	S. 37	6·27	4·86	6·72	5·82	6·39
	S. 19	6·35	5·27	6·54	6·09	6·65
	Y. 37	6·17	4·26	5·86	4·53	5·80
	Y. 19	6·24	4·36	5·36	4·64	5·38
L. fermenti: R. 1	S. 37	6·48	5·24	5·15	4·84	5·10
	S. 19	6·45	6·26	N.D.	5·74	6·17
	Y. 37	6·27	3·85	5·35	3·84	5·48
	Y. 19	6·39	4·83	5·36	4·94	5·21
L. pentoaceticus: W. 1	S. 37	6·14	4·35	—	5·16	—
	S. 19	6·33	5·42	—	6·24	—
	Y. 37	6·25	4·33	—	4·94	—
	Y. 19	6·13	4·64	—	5·39	—
W. 2	S. 37	6·24	4·83	5·93	4·87	5·63
	S. 19	—	—	—	—	—
	Y. 37	6·26	4·77	5·63	4·75	5·40
	Y. 19	—	—	—	—	—

		Control	Ae.	Ae.T.	An.	An.T.
L. buchneri: P. 3	S. 32, S. 19	No significant growth				
	Y. 32	N.D.	N.D.	N.D.	N.D.	N.D.
	Y. 19	6·10	3·95	3·95	3·95	3·95
L. pentoaceticus: L. 28	S. 37, S. 19	No significant growth				
	Y. 37	6·02	4·16	4·22	4·44	4·24
	Y. 19	6·20	4·16	4·10	4·38	4·23
947	S. 37, S. 19	No significant growth				
	Y. 37	6·15	4·23	4·21	4·46	4·39
	Y. 19	6·29	3·86	3·81	4·28	3·85
L. pastorianus: Shim.	S. 32, S. 19	No significant growth				
	Y. 32	6·32	4·26	4·17	4·78	4·38
	Y. 19	6·25	4·21	4·11	4·47	4·36

Ae. = aerobic. An. = anaerobic. C. = chalk. T. = talc. S. = separated milk.
Y. = yeast separated milk. 19, 25, 32, 37 = ° C. N.D. = not done.
Dashes indicate where series of experiments have been omitted.

The lowest values range from 3·55 to 3·84 (2797, R. 1, 947, W. 5). The lowest *p*H induced by strain 4034 is 4·26, but all other strains were capable of growth below *p*H 4·20. Comparing these values with those for the streptococci (lowest *p*H values attained 4·10–4·75 (1)) it may be seen that in general the lactobacilli can grow at a lower *p*H, which is in agreement with the findings of Davis & Thiel (10), Orla-Jensen (2) and Pederson (4).

The *p*H values of the controls were lower after incubation for 4 weeks at 37° than after 8 weeks at 19°. The maximum difference recorded is 0·19 of a *p*H unit.

Sørensen nitrogen titrations

In general a small decrease in the Sørensen titration occurred except in a few cases where significant increases (20–50 %) were recorded for cultures containing chalk (strains L. 28, W. 2, W. 1, R. 1 and 1724).

General

As was previously recorded when dealing with the streptococci (1), the greatest single difference between the homofermentative and heterofermentative groups is the presence in the heterofermentative types of enzymes permitting alcoholic fermentation, and that about molecular equivalents of alcohol and lactic acid are produced. In addition the heterofermentative lactobacilli show similar behaviour to the analogous streptococcal group in that the Pasteur effect is slight: alcoholic fermentation proceeds almost as well under "aerobic" as under "anaerobic" conditions. Respiration, therefore, is of secondary importance compared with fermentation in the supply of energy, although from the point of view of flavour in dairy products it would be dangerous to neglect the respiratory activity of these organisms. As regards the amount of acetic acid formed, this is always small compared with the amount of alcohol, and it appears that an estimation of the amount of carbon dioxide or alcohol produced is a better guide to the classification of such types than volatile acid estimation.

SUMMARY

1. The influence of temperature, oxygen tension, yeast autolysate and chalk on the production of by-products and the ratios of by-products formed to sugar utilized and lactic acid produced in milk by the lactobacilli has been investigated.

2. The total production of lactic acid is increased by the presence of chalk and "growth factors" but is not affected by temperature or oxygen tension. The ratio of lactic acid produced to sugar utilized is decreased at lower temperatures and increased by chalk but yeast autolysate and variation in oxygen tension give variable results.

3. The total production of acetic acid is increased by the presence of chalk, and "growth factors" and by anaerobic conditions. The ratios of acetic acid produced to sugar utilized and lactic acid formed are decreased by chalk

and anaerobic conditions and there is a tendency for the ratios to be decreased by the presence of yeast and increased at lower temperatures.

4. The total production of alcohol is increased by anaerobiosis and also by the presence of chalk and yeast in the medium. The ratios of alcohol formed to sugar utilized and lactic acid produced is increased by anaerobic conditions, and chalk also tends to increase the relative amount of alcohol. Temperature and yeast autolysate have little effect.

5. Chalk is effective in maintaining the *p*H at a higher level during growth, but the influence of chalk on the ratios of by-products formed to sugar utilized is probably not entirely a *p*H effect since the addition of talc to the medium has an equally strong influence in altering the relative production of lactic acid and alcohol.

6. It was previously shown for the heterofermentative streptococci (1) that the Pasteur effect (suppression of alcoholic fermentation by respiration) is slight; similar results have been obtained with the heterofermentative lactobacilli.

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258. THE VITAMIN B_1 AND RIBOFLAVIN OF MILK

PART I. THE APPLICATION OF JANSEN'S THIOCHROME TEST TO THE ESTIMATION OF VITAMIN B_1 IN MILK

BY J. HOUSTON, S. K. KON AND S. Y. THOMPSON

PART II. THE DIFFERENT FORMS OF VITAMIN B_1 IN MILK

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PART III. EFFECT OF STAGE OF LACTATION AND OF SEASON ON THE VITAMIN B_1 AND RIBOFLAVIN CONTENT OF MILK

BY J. HOUSTON, S. K. KON AND S. Y. THOMPSON

PART IV. COMPARISON OF CHEMICAL AND BIOLOGICAL METHODS OF ESTIMATION OF VITAMIN B_1

BY K. M. HENRY, J. HOUSTON, S. K. KON AND P. WHITE

From the National Institute for Research in Dairying, University of Reading

(With 5 Figures)

SYNOPSIS

Part I. *The application of Jansen's thiochrome test to the estimation of vitamin B_1 in milk.*

Introduction. Experimental. The thiochrome test. Study of factors influencing the action of takaphosphatase (takadiastase) on milk in the chemical estimation of vitamin B_1. Effect of *p*H. Period of incubation. Method of incubation. Peptic digestion. Tryptic digestion. Comparison of the effect of takaphosphatase, pepsin and trypsin preparations on the assay of vitamin B_1 in milk. The effect of crystalline pepsin.

Part II. *The different forms of vitamin B_1 in milk.*

Fractionation of the vitamin B_1 of milk. Introduction. Ultra-filtration through Cellophane. Ultra-filtration through collodion membranes. The extraction of vitamin B_1 from milk by trichloroacetic acid. Examination of the protein residue obtained in the ultra-filtration of milk through Cellophane. Vitamin B_1 firmly bound to protein. Trichloroacetic acid experiments. Fuller's earth adsorbates. Form in which cocarboxylase is present in full lactation milk.

Part III. *Effect of stage of lactation and of season on the vitamin B_1 and riboflavin content of milk.*

Vitamin B_1. Stage of lactation. Study of individual cows. Correlation between "free" vitamin B_1 and the phosphomonoesterase A_1 titre of milk. The cocarboxylase-splitting enzymes of milk. "Free" and phosphorylated vitamin B_1 in the milk of the goat. Effect of frequent milkings on the partition of vitamin B_1 in milk. The source of the vitamin B_1 of milk. Seasonal changes. First experiment. Second experiment. Results. Riboflavin. Stage of lactation. Seasonal changes. First experiment. Second experiment.

Part IV. *Comparison of chemical and biological methods of estimation of vitamin B_1.*

Introduction. Experimental. First experiment (October 1937). Second experiment (June 1938). Third experiment (January 1939). Fourth experiment (April 1939). Fifth experiment (April 1939). Sixth experiment (June 1939). Seventh experiment (October 1939). Statistical analysis.

Summary.

PART I. THE APPLICATION OF JANSEN'S THIOCHROME TEST TO THE ESTIMATION OF VITAMIN B_1 IN MILK

A. Introduction

The development by Jansen (1) of a satisfactory technique for the chemical estimation of vitamin B_1 (aneurin) has been of the greatest importance for work on the natural distribution of this factor. We were interested in the application of this test to milk and milk products and used it soon after the method became available.

From preliminary experiments (2) it quickly became evident that with milk the thiochrome test gave lower values than the biological assay and it became necessary to investigate further both the biological technique and the chemical method.

Kinnersley & Peters (3,4) have shown that the thiochrome of cocarboxylase does not pass into the isobutanol layer in the Jansen technique. At first we did not consider that the low results obtained by the chemical test were due to failure to estimate cocarboxylase, as in most cases only a slight fluorescence could be seen in the watery layer. It occurred to us later that cocarboxylase might be present in a combined form and that vitamin B_1 in milk might be joined to protein and so not react in the test.

We decided, therefore, to digest the milk with pepsin in order to break down possible protein complexes. In addition, on the advice of Prof. Peters and Prof. Kay, we incubated milk with takadiastase, since Kinnersley & Peters (5) had shown that this preparation (which contains takaphosphatase in addition to many other enzymes) is capable of hydrolysing quantitatively cocarboxylase to vitamin B_1 at *p*H 4, i.e. at a *p*H at which vitamin B_1 is quite stable.

B. Experimental

(1) *The thiochrome test*

After many preliminary tests the Jansen method was applied to milk in the following form.

A series of three sets of glass-stoppered bottles was set up in duplicate, each containing 6 ml. of milk; 1 ml. of methanol was added to each and 0·20 and 0·25 ml. of a 1 % solution of $K_3Fe(CN)_6$ was added to the first and second flask respectively, the third sample being kept as a "blank". 3 ml. of a 40 % solution of NaOH was added to each and, after 1 min., 13 ml. of redistilled isobutanol. The fluorescence of 10 ml. of the isobutanol layer was measured in a Cohen fluorimeter (6), using the outfit already described by us for riboflavin measurements (7).[1] The higher of the two measurements was used for calculating the vitamin B_1 content. The "blank" usually contained only traces of fluorescence.

[1] For vitamin B_1 readings the Wratten gelatine filter no. 12 was omitted and a quinine sulphate solution calibrated against crystalline vitamin B_1 was used as standard.

In most bulked milks fluorescence in the aqueous layer is faint and difficult to see and fades on standing for about 1 hr. In some processed milks, e.g. evaporated milk and sterilized milk, there is usually a very intense fluorescence in the aqueous layer but this also appears in the "blank". In some protein fractions (*vide infra*) a blue fluorescence was observed in the test flasks but not in the blank, although it was subsequently found that this fluorescence was not due to cocarboxylase. It is evident, therefore, that reliable cocarboxylase estimations cannot be made on the aqueous layers of milks. Cocarboxylase can, however, be measured in the aqueous layers of milk sera and a reliable measure of cocarboxylase is obtained by takaphosphatase treatment.

At first the fluorimeter was standardized against the international clay standard for vitamin B_1 but later (after June 1938, cf. p. 167) the crystalline vitamin B_1 standard was used.

When the thiochrome test was applied in this form to milk it gave low results (2). Incubation with enzymes was then tried and a marked increase in fluorimetric readings was obtained. The best conditions for the enzyme action were then studied.

(2) *Study of factors influencing the action of takaphosphatase (takadiastase) on milk in the chemical estimation of vitamin B_1*

(*a*) *Effect of* p*H.*

A sample of raw milk which had been heated to 70° C. for 5 min. to destroy the natural enzymes was divided into five lots of 100 ml. and each was brought to a *p*H value as shown in the table. After the addition of takadiastase (Parke, Davis and Co.), all milks were incubated at 37° C. for 6 hr. The results are given in Table 1.

Table 1. *Incubation of milk with takadiastase at different* p*H*

*p*H	Vitamin B_1 in μg./100 ml.
Original milk	19·5
6·48	24·6
5·62	28·2
5·00	30·3
4·10	30·0
3·20	25·2

The reaction goes best at *p*H 4–5, the optimal range of takaphosphatase. This may mean that of the enzymes present in takadiastase, this enzyme is chiefly responsible for the increase in vitamin B_1, but other effects cannot be excluded.

(*b*) *Period of incubation.*

A large bulk of milk was incubated at *p*H 3·9 with takaphosphatase (takadiastase), samples being taken at the times indicated. The milk had previously been heated to 70° C. for 5 min. The results are given in Table 2.

Table 2. *Effect of period of incubation with takaphosphatase on the vitamin B_1 assay of milk*

Period of incubation hr.	Vitamin B_1 in μg./100 ml.
0	18·9
½	35·7
1	36·6
4	37·2
6	38·0
18	30·9
24	33·9
48	32·7

It will be seen that a large increase is obtained even after ½ hr., while the maximum is reached after 4–6 hr.

(*c*) *Method of incubation.*

Following these findings, the incubation of milk with takadiastase was carried out thus: 100 ml. of milk were heated to 70° C. for 5 min. to destroy the natural enzymes, and were rapidly cooled.[1] 240 mg. of takadiastase (Parke, Davis and Co.) were added and thoroughly shaken. 5 ml. of approximately *N* HCl were slowly added with constant shaking. This brought the *p*H to approximately 4·0; the *p*H was checked. After addition of a few drops of chloroform the whole was incubated for from 3 to 6 hr. at 37° C. The Jansen method, as described above, was applied to all milks before and after incubation.

A typical result of incubation with takaphosphatase is given in Table 3.

Table 3. *Effect of incubation of milk with takaphosphatase on the fluorimetric assay of vitamin B_1*

Date	Description of milk	Vitamin B_1 in μg./100 ml.
20. x. 38	Bulk milk heated to 70° C. for 5 min.	25·1
	Same milk incubated at *p*H 4 for 3 hr.	24·1
	Same milk incubated with takaphosphatase for 3 hr.	52·0

After takaphosphatase incubation the value was doubled (Table 3) suggesting that the agreement between chemical and biological tests would now be more satisfactory. This problem is discussed further in Part IV of this paper (8).

(3) *Peptic digestion*

For peptic digestion removal of fat appeared advisable. 500 ml. of separated milk were incubated at 45° C. with 2·5 g. of commercial (B.D.H.) pepsin at a *p*H of about 2 for 2 days. The *p*H was readjusted after the first 24 hr. and 2·5 g. of fresh pepsin were then added. The pepsin preparation contained only traces of vitamin B_1 but generally gave a high "blank" value.

[1] The heating was used only in investigations of the state of vitamin B_1 in milk. It is unnecessary in routine estimations of vitamin content.

(4) *Tryptic digestion*

Tryptic digests were also carried out on 500 ml. of separated milk, incubation being carried out at *p*H 6 and at 38° C. for 2 days. 2·5 g. of commercial (B.D.H.) trypsin were used and another 2·5 g. were added after the first 24 hr. Peptic and tryptic digestion were followed by incubation with takaphosphatase. For this purpose the *p*H was readjusted to 4 and the process carried out as already described.

The tests can equally well be applied to dried and evaporated milks which, for this purpose, are reconstituted with water to the original volume.

(5) *Comparison of the effect of takaphosphatase, pepsin and trypsin preparations on the assay of vitamin B_1 in milk*

Table 4 shows the change in the vitamin B_1 assay of milk after enzyme treatment.

Table 4. *Effect of treatment with takaphosphatase, pepsin and trypsin on the vitamin B_1 assay of milk*

Treatment	Vitamin B_1 in μg./100 ml.
Bulked separated milk heated to 70° C. for 5 min.	18·9
Same after peptic digestion	25·8
Same after peptic digestion followed by takaphosphatase	35·4
Same after tryptic digestion	24·0
Same after tryptic digestion followed by takaphosphatase	31·5
Same after takaphosphatase only	31·2

It will be seen that in this case the greatest increase was in the milk treated successively with pepsin and takaphosphatase. This is not always the case, and Table 5 shows that this preliminary pepsin treatment can be omitted without causing an appreciable error, with the possible exception of late lactation milks. In view of the time involved in the peptic digestion, only the incubation with takaphosphatase is used in our routine tests.

Table 5. *Comparison of vitamin B_1 values obtained on various milks directly (D), after takaphosphatase treatment alone (T), or following peptic digestion (PT), or after peptic digestion alone (P)*

Vitamin B_1 in μg./100 ml. All values on fat-free samples.

Date	Type of milk	Vitamin B_1 D	P	P – D	T	PT	PT – T
28. vi. 39	Early lactation	9·6	14·4	4·8	54·3	55·8	1·5
14. ii. 39	Bulk milk	24·3	32·1	7·8	36·6	39·6	3·0
22. vi. 39	Mid-lactation	20·1	24·6	4·5	28·8	29·1	0·3
22. vi. 39	Mid-lactation	27·9	29·7	1·8	32·1	30·1	−2·0
2. ii. 39	Sep. spray dried (1)	16·8	26·4	9·6	35·4	37·2	1·8
2. ii. 39	Sep. spray dried (2)	21·4	26·9	5·5	41·0	41·1	0·1
2. ii. 39	Sep. roller dried	18·3	26·0	7·7	29·0	31·2	2·2
2. ii. 39	Sep. sterilized	12·2	16·7	4·5	22·8	22·7	−0·1
1. ii. 40	Late lactation (1)	35·6	41·6	6·0	35·2	42·0	6·8
12. ii. 40	Late lactation (2)	34·3	37·5	3·2	31·8	37·9	6·1
12. ii. 40	Late lactation (3)	31·5	36·3	4·8	32·2	37·5	5·3
Mean		22·9	28·4	5·5	34·5	36·8	2·3
Mean, omitting late lactation figures		18·8	24·6	5·8	35·0	35·9	0·9

Both the peptic and tryptic treatments caused an increase in the vitamin B_1 measurement (some 5–6 μg. in this case, Table 4), but there still remained about 10 μg. which was not liberated by either treatment but was set free by taka-phosphatase. It is likely that the first increase was due to the liberation of aneurin from a protein complex. As shown below, it was not due to splitting of cocarboxylase which probably followed takaphosphatase treatment. The commercial pepsin preparation doubtless contained other enzymes.

(6) *The effect of crystalline pepsin*

Through the kindness of Dr J. Philpot of Oxford a very pure preparation of pepsin was obtained and its action was compared with that of the impure product. A preparation of cocarboxylase, given to Dr J. G. Davis by Prof. Lohmann, was used in these experiments. We found that this preparation contained no free vitamin B_1 and that 1 μg. yielded 0·65 μg. of vitamin B_1. The results are set out in Table 6.

Table 6. *Effect of pure and of commercial pepsin on the vitamin B_1 assay in milk with or without added cocarboxylase. Bulk separated milk (heated for 5 min. to 70° C.) was used*

Treatment	Vitamin B_1 in μg./100 ml.
Milk before incubation	22·3
After takaphosphatase	36·4
Peptic* digestion alone	35·8
Peptic* digestion followed by takaphosphatase	38·4
Peptic* digestion alone‡	30·8
Peptic* digestion followed by takaphosphatase‡	55·1
Peptic† digestion alone	32·7
Peptic† digestion followed by takaphosphatase	37·5
Peptic† digestion alone‡	32·7
Peptic† digestion followed by takaphosphatase‡	55·0

* Pure pepsin.

† Commercial pepsin.

‡ Cocarboxylase equivalent to 16·5 μg. vitamin B_1 was added to the milk.

It is evident from the table that neither commercial pepsin nor the pure preparation cause, in heated milk, any conversion of cocarboxylase to vitamin B_1 at *p*H 2 and 45° C. Separate tests have also shown that this does not occur at *p*H 4 at 37° C. Peptic digestion is of interest as it allows an estimation of free and bound vitamin B_1 in milk as distinct from free or bound cocarboxylase.

Numerous tests have shown that aneurin added to the different digestion mixtures was recovered quantitatively and that there was most probably no loss of the vitamin B_1 of milk in the course of such procedures. Good agreement between biological test and fluorimetric measurements on incubated milk is reported in Part IV of this paper.

PART II. THE DIFFERENT FORMS OF VITAMIN B_1 IN MILK

A. FRACTIONATION OF THE VITAMIN B_1 OF MILK

(1) *Introduction*

The liberation of a part of vitamin B_1 of milk by pepsin and trypsin suggested the presence of vitamin B_1-protein complexes. This point was studied by subjecting milk to ultra-filtration. Protein-free milk serum was obtained by high-pressure ultra-filtration through No. 200 Cellophane by means of the apparatus of Folley & Mattick (9), and by low-pressure ultra-filtration through collodion membranes in the apparatus described by Folley (10). Treatment of milk with trichloroacetic acid by the method of Hennessy & Cerecedo (11) was also studied.

(2) *Ultra-filtration through Cellophane*

25 ml. lots of fat-free milk were filtered at pressures of 60–120 atm. The process usually took about 12 hr. Separated milk was used and was heated to 70° C. for 5 min. before filtration.[1] A few drops of ether were added to check bacterial growth. Water-clear filtrates were obtained from which nothing could be precipitated with 20 % trichloroacetic acid even on warming. The protein was washed under pressure twice with 10 ml. of distilled water, the washings being combined with the original serum. The washings also took some 12 hr. each. The protein was dried *in vacuo* over calcium chloride, ground and redissolved in 25 ml. of water. Both serum and protein were tested for vitamin B_1 before and after incubation with takaphosphatase. Peptic digests were also made on the protein. The results are given in Tables 7 and 10.

(3) *Ultra-filtration through collodion membranes*

The membranes were prepared and filtration was carried out as described by Folley (10). Six tubes, each containing 10 ml. of milk, were used and filtration was carried out for periods of up to 15 hr. A protein fraction could not be obtained by this method. The results are given in Table 7.

It at once becomes clear that up to 50 % of the vitamin B_1 measured prior to incubation in the original milk does not pass through the Cellophane. Since added aneurin passes quantitatively through the filter, it must be concluded that this portion is in some way combined with the protein. We have verified that the difference is not due to adsorption of vitamin B_1 on Cellophane or on the supporting filter paper. It will be seen later that the missing fraction can be quantitatively recovered from the protein.

Another point of interest is the fact that only with colostrum or early milk was the vitamin B_1 content of serum increased appreciably after incubation

[1] As ultra-filtration takes a considerable time, this heat treatment was necessary to avoid enzyme action. The experience of Sinclair (12) with blood shows that loose vitamin B_1-protein complexes may be split by this procedure. However, an ultra-filtration carried out as aseptically as possible at 0° C. on milk not subjected to heat treatment yielded results similar to those obtained by the routine method.

with takaphosphatase. Since added cocarboxylase is not retained by the filter, this would prove that in normal milk the cocarboxylase, when present, is bound to larger complexes.

Table 7. *Distribution of vitamin B_1 in milk as studied by ultra-filtration through Cellophane and through collodion membranes*

Vitamin B_1 in μg./100 ml.

Type of milk	No.	Milk		Milk serum	
		Before incubation	After incubation	Before incubation	After incubation
		Cellophane			
Bulk milk	1	24·3	36·6	18·3	18·9
	2	17·4	31·8	7·5	7·8
	3	20·1	28·8	14·1	13·5
Reconstituted spray-dried separated milk	4	21·9	38·1	14·1	15·9
	5	21·0	31·2	12·0	14·4
	5*	70·8	78·9	62·4	59·1
	5†	21·9	61·8	10·7	39·9
Colostrum	6	6·3	55·5	4·0	27·3
Early milk	7	9·6	54·3	4·2	12·2
		Collodion			
Reconstituted spray-dried separated milk	5	21·0	31·2	13·7	17·9
Early milk	8	24·2	35·0	15·0	27·3
Late milk	9	31·2	31·5	26·7	27·6

* 50 μg./100 ml. of aneurin added.

† Cocarboxylase added equivalent to 31·8 μg. of vitamin B_1 per 100 ml.

Table 7 shows that an essentially similar distribution of vitamin B_1 can also be obtained by means of collodion filters, though a somewhat greater proportion of vitamin B_1 and of the substance yielding vitamin B_1 on incubation with takaphosphatase passes through the filter.

(4) *The extraction of vitamin B_1 from milk by trichloroacetic acid*

A method for the estimation of vitamin B_1 involving extraction with trichloroacetic acid has been described by Hennessy & Cerecedo (11). We have applied their method of extraction to milk in the following way. 100 ml. of separated milk were boiled under reflux for 3 min. with 70 ml. of 2 % trichloroacetic acid and were then centrifuged. The protein fraction was stirred with 30 ml. of 2 % trichloroacetic acid and, after centrifuging, the extracts were combined. The resulting fluid had a *p*H of about 4·0. Vitamin B_1 estimations were carried out directly on the combined extracts before and after incubation with takaphosphatase. The protein fraction was extracted with ether, suspended in water and digested with pepsin. Table 8 shows results obtained on trichloroacetic acid serum compared with those obtained by the usual method on the original milk.

Table 8. *Comparison of the vitamin B_1 content of milk and of trichloroacetic acid serum from milk before (D) and after incubation with takaphosphatase (T). Values for the protein residue after trichloroacetic acid treatment before (D) and after digestion with pepsin (P) and treatment with takaphosphatase (PT)*

Vitamin B_1 in μg./100 ml.

				Trichloroacetic acid treatment					
		Milk		Serum			Protein		
Date	Type of milk	D	T	D	T	Milk T – Serum T	D	P	PT
13. vi. 39	1st colostrum	36·6	65·4	33·0	66·3	−0·9	5·0	12·8	19·1
17. vi. 39	4th colostrum	6·3	60·0	6·6	63·9	−3·9	—	4·4	5·6
13. vi. 39	Early lactation	9·3	36·0	9·3	36·3	−0·3	0·84	3·7	4·0
24. v. 39	Early lactation	6·6	49·5	5·8	48·9	+0·6	0·51	2·5	2·9
30. v. 39	Early lactation	9·6	44·1	6·6	42·9	+1·2	—	2·4	6·0
13. vi. 39	Early lactation	10·2	39·6	8·4	37·5	+2·1	1·3	2·7	4·3
28. vi. 39	Early lactation	9·6	54·3	9·9	48·0	+6·3	—	3·6	6·0
18. iii. 39	Bulk milk	21·9	34·2	23·4	25·5	+8·7	—	1·3	7·5
26. vi. 39	Bulk milk	17·4	31·8	18·0	30·9	+0·9	—	3·6	5·1
21. i. 40	Bulk milk*	27·1	52·5	26·8	48·8	+3·7	0·83	3·8	5·0
22. vi. 39	Mid-lactation	27·9	32·1	22·3	27·9	+4·2	0·33	2·7	3·6
22. vi. 39	Mid-lactation	20·1	28·8	19·2	25·0	+3·8	0·66	3·0	3·4
2. vi. 39	Mid-lactation	20·7	28·2	14·1	23·0	+5·2	—	5·2	6·0
9. vi. 39	Mid-lactation	23·9	32·1	16·8	26·4	+5·7	—	5·3	5·7
3. vii. 39	Separated spray-dried milk	21·0	31·2	20·5	29·7	+1·5	—	4·2	4·2
3. vii. 39	Same but 50 μg. of vitamin B_1 added	70·8	78·9	69·6	78·3	+0·6	—	4·5	4·5
12. ii. 40	Late lactation	31·5	32·2	29·2	31·3	+0·9	0·83	3·6	4·0
12. ii. 40	Late lactation	34·3	31·8	29·3	26·0	+5·8	0·83	3·8	3·6
Mean		22·5	42·4	20·5	39·8	+2·6	—	4·1	5·6
Mean omitting colostrum		22·6	39·8	20·6	36·6	+3·2	—	3·5	4·7

* Includes many freshly calved cows.

The agreement is on the whole quite good. With the exception, however, of colostrum the figures obtained by the usual method are slightly but uniformly higher than those obtained by the trichloroacetic acid method. The difference amounts to some 3 μg./100 ml. or about 10 %. This quantity of vitamin B_1 can be quantitatively recovered from the trichloroacetic acid protein as is evident from the table. The agreement between the values obtained before incubation in serum and milk shows that trichloroacetic acid extracts some of the vitamin B_1 retained by ultra-filters. This is also shown by the direct comparison in Table 9. We have also found that the trichloroacetic acid extract passes quantitatively through the Cellophane ultra-filter.

Table 9. *Comparison of ultra-filtrate and trichloroacetic acid sera*

Vitamin B_1 in μg./100 ml. Separated spray-dried milk used.

Original milk		Cellophane ultra-filtrate		CCl_3COOH filtrate	
Before incubation	After incubation	Before incubation	After incubation	Before incubation	After incubation
21·2	31·2	12·0	14·4	20·5	29·7

(5) *Examination of the protein residue obtained in the ultra-filtration of milk through Cellophane*

Vitamin B_1 was estimated before and after enzymic treatment in the protein fraction of milk ultra-filtrates and a balance sheet showing the partition of vitamin B_1 in milk is given in Table 10. Ultra-filtrations of several of 25 ml. portions of milk were necessary to provide enough protein.

Table 10. *The distribution of vitamin B_1 in milk*

Vitamin B_1 in μg./100 ml.

Substrate	Treatment	Result 1*	Result 2†
Original milk	Before incubation	21·9	24·3
	After incubation with takaphosphatase	38·1	36·6
	After incubation with pepsin	32·4	32·1
	After incubation with pepsin followed by takaphosphatase	36·6	39·6
Ultra-filtrate	Before incubation	14·1	18·3
	After incubation with takaphosphatase	15·9	18·9
Protein	Before incubation	6·9	6·9
	After incubation with takaphosphatase	16·8	12·6
	After incubation with pepsin	11·1	9·9
	After incubation with pepsin followed by takaphosphatase	17·7	13·5

* Spray-dried separated milk. † Separated bulk milk.

Other estimations not included in the table were carried out and it was found that from 6 to 9 μg. of vitamin B_1 could always be demonstrated in the protein fraction by the thiochrome test prior to incubation. This fraction is probably only loosely bound. Together with the vitamin B_1 directly measurable in the ultra-filtrate it is equivalent to the direct reading on the original milk. Figures in Table 8 show that this loose combination is also broken up by the action of trichloroacetic acid. It will be seen further that the combined values for ultra-filtrate and protein after incubation account almost quantitatively for the amount found in the original milk.

(6) *Vitamin B_1 firmly bound to protein*

(*a*) *Trichloroacetic acid experiments.*

The increase after peptic digestion of the protein retained by Cellophane is similar to that observed in milk after pepsin treatment (Tables 4 and 5) and shows that part of the vitamin B_1 is firmly bound to protein. Presumably the same fraction is found on the protein residue after trichloroacetic acid extraction. In Tables 5 and 8 the increases in vitamin B_1 titration after peptic digestion of milk and of the trichloroacetic acid residue, are set out.

The amounts liberated from the ultra-filtrate residue were (Table 10) 4·2 and 3·0 μg./100 ml. and there is no doubt that in all three cases the figures are of the same order. It appears therefore that in the milks studied about 5 μg./100 ml. of vitamin B_1 is firmly bound to protein. This fraction is not liberated by trichloroacetic acid extraction and is therefore not measured by the method of Hennessy & Cerecedo (11).

(*b*) *Fuller's earth adsorbates.*

Similar results were obtained when milks were exhaustively extracted with adsorbent fuller's earth after the manner of Wiegand (13). For this purpose 50 ml. of separated milk were diluted 1:4 with water and shaken three times with 1 g. of a neutral sample of fuller's earth (B.D.H.) for each extraction. Vitamin B_1 estimations were then carried out on the remaining liquid. 2 μg./100 ml. of vitamin B_1 were found to be present in the "free"[1] form but this value rose to 7 μg./100 ml. after treatment with pepsin or takaphosphatase. Undiluted milk gave substantially similar values. Here again some 5 μg./100 ml. appear to be firmly bound to protein.

B. Form in which cocarboxylase is present in full lactation milk

It has been shown that the ultra-filtrate serum of full lactation milk contained no cocarboxylase, though incubation with takaphosphatase brought about an increase in the original milk. Hence one would expect that the cocarboxylase was in combination with the protein. Table 10 shows that this is actually the case. Since, however, most of this cocarboxylase is split off by refluxing with 2 % trichloroacetic acid, it is probably less firmly bound than the fraction of vitamin B_1 which cannot be removed from the protein precipitate by this process. Table 8 shows that in agreement with this view there is, for the majority of milks, only a very small rise in the vitamin B_1 content of the trichloroacetic acid protein precipitate when this is subjected to further incubation with takaphosphatase following peptic digestion. For some milks, however, the increment is outside the limits of experimental error.

PART III. EFFECT OF STAGE OF LACTATION AND OF SEASON ON THE VITAMIN B_1 AND RIBOFLAVIN CONTENT OF MILK

A. Vitamin B_1

(1) *Stage of lactation*

(*a*) *Study of individual cows.*

It has already been mentioned in Part II of this paper that, as far as vitamin B_1 is concerned, colostral and early milks differ from full lactation milks in two respects: they contain large quantities of cocarboxylase and their total vitamin B_1 content is higher. To study this matter more closely the milks of six Shorthorn and of one Guernsey cow were studied from parturition throughout lactation. The cows had calved between March and July. In addition the milks of groups of Shorthorn cows, of which one was kept indoors throughout the experiment on a winter ration while the other had the same treatment as the herd and had normal access to pasture, were examined for 9 months at frequent intervals beginning between the first and third month

[1] "Free" refers to the vitamin B_1 directly measured by the Jansen technique. Some of it is without doubt combined with larger complexes, cf. p. 151 and Tables 7 and 10.

of lactation. The results are given later in this paper (p. 164). Values for free and total vitamin B_1 for the seven cows studied individually are given in Fig. 1. In all cases where the examination of colostrum was started immediately after parturition the following changes could be observed: in the first day's colostrum the ratio of "free" to total vitamin B_1 was similar to that of full lactation milk but the total content was slightly higher. A very marked change took place in the succeeding one or two days, characterized by a sharp drop in the "free" vitamin B_1 and, as a rule, a corresponding increase in the total. At the same time, in the thiochrome test, unmistakably strong fluorescence characteristic of cocarboxylase could be seen in the aqueous layer after isobutanol extraction. After that the "free" vitamin B_1 gradually rose while the total declined and the curves got closer and tended to meet as lactation progressed. A similar picture will be found in the results obtained on the bulked milk of the "indoor" and "outdoor" cows (Fig. 3).

It will be noticed from Tables 5 and 8 and Fig. 1 that in some late lactation milks incubation with takaphosphatase of milk, milk trichloroacetic acid sera or protein yields figures *lower* than before incubation. We do not know the reason for this phenomenon.

(*b*) *Correlation between "free" vitamin B_1 and the phosphomonoesterase A_1 titre of milk.*

The nature of the changes in "free" vitamin B_1 is very reminiscent of the alterations in the alkaline phosphomonoesterase titre of milk with progressing lactation observed by Folley & Kay (14). According to them this enzyme is also high immediately after parturition; it then falls suddenly to rise gradually again. Estimations of the phosphomonoesterase content, expressed in King & Armstrong (15) units, were carried out on samples of the colostrum and milk of the seven cows studied. They are plotted in Fig. 1, and it is evident that there is a close relation between "free" aneurin and the enzyme titre. This will be more plainly seen from Table 11 which gives mean phosphomonoesterase figures for progressing lactation and the corresponding mean content of "free" vitamin B_1 expressed as a percentage of the total for the seven cows studied individually. On the other hand, the fraction which can be measured only after incubation with takaphosphatase, presumably largely cocarboxylase, varies inversely with alterations in phosphomonoesterase. Table 12, which gives the approximate distribution of different forms of vitamin B_1 in milk throughout lactation, further illustrates this point. Such a correlation between a dephosphorylating enzyme, the phosphorylated substrate and the phosphorus-free end-product would naturally be expected, but in this case the relationship cannot for two reasons be so direct or simple. In the first place the phosphatase measured was the phosphomonoesterase A_1 of Folley & Kay (16), the optimal activity of which lies at a *p*H of about 9, well above the natural *p*H of milk. In the second place cocarboxylase is a pyrophosphate on which this enzyme, even at its optimal *p*H, would not be expected to act at all. It there-

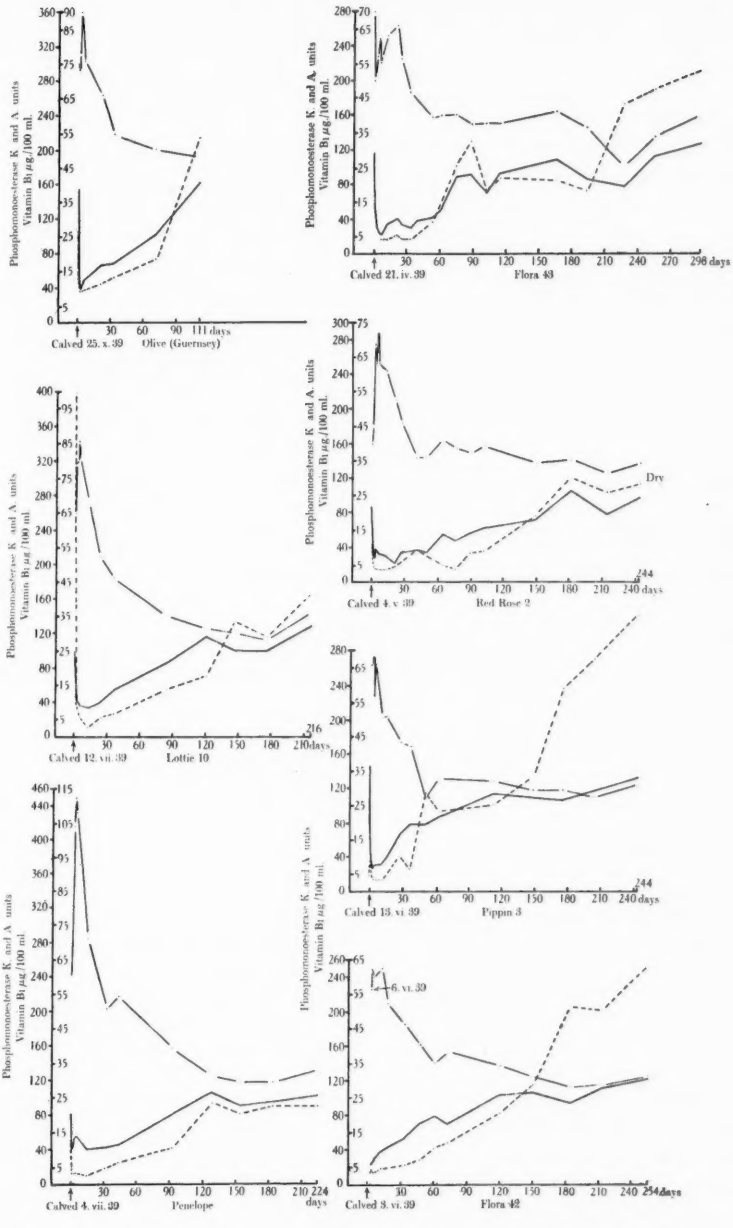

Fig. 1. "Free" and total vitamin B_1 and phosphatase content of the milk of six Shorthorn and one Guernsey cow in the course of lactation.

—·— Total vitamin B_1. ——— "Free" vitamin B_1. – – – – Phosphomonoesterase.

Table 11. *Correlation between "free" vitamin B_1 expressed as a percentage of the total and the phosphomonoesterase titre of the milk of seven cows in progressing lactation*

No. of observations	"Free" vitamin B_1 as percentage of total, mean and range	Phosphomonoesterase titre, mean and range
	Phosphomonoesterase in King and Armstrong units	
30	14·0 (9·4–25·7)	16·6 (9·0–44·9)
16	34·6 (25·8–47·1)	33·6 (17·6–71·6)
16	61·8 (51·1–74·8)	87·2 (42·0–129·2)
15	86·7 (76·1–100)	151·6 (80·3–270·0)

Table 12. *Approximate distribution of different forms of vitamin B_1 in milk throughout lactation*

Type of milk	Colostrum*	Early milk	Mid-lactation milk	Late lactation milk
Phosphomonoesterase in King and Armstrong units	<20	About 20	80–120	150–300
Total amount (*a*) (μg./100 ml.) ...	60–100	About 60	30–35	30–40
	Unphosphorylated			
Bound to protein:				
Diffusible (*b*) % of total	6	8	49	65
Loose combination (*c*) % of total	4 } 17	10 } 25	21 } 80	25 } 100
Firmly bound (*d*) % of total	7	7	10	10
	Phosphorylated			
Bound to protein:				
Diffusible (*e*) % of total	40	15	0	0
Loose combination (*f*) % of total	40 } 82	57 } 76	20 } 21	0 } 0
Firmly bound (*g*) % of total	2	4	1	0

* Excluding the first colostrum.

(*a*) After incubation of milk with takaphosphatase.

(*b*) Passing through Cellophane.

(*c*) Difference between direct estimation in milk and (*b*).

(*d*) Difference between measurement after peptic digestion and direct estimation.

(*e*) Increase on takaphosphatase treatment of ultra-filtrate serum.

(*f*) Increase on takaphosphatase treatment of trichloroacetic acid serum *less* (*e*) (cf. p. 155).

(*g*) Difference between measurement on ultra-filtrate protein after digestion with pepsin and takaphosphatase and peptic treatment alone.

fore appears that other enzymes more specifically suited to the dephosphorylation of cocarboxylase must accompany in milk this phosphomonoesterase. Kay (17) has shown that a pyrophosphatase is widely distributed in mammalian tissues and that its distribution is similar to that of the orthophosphoric esterase (phosphomonoesterase), but that its zone of optimal activity is between *p*H 7·2 and 7·8. Table 1 of Kay's paper (17) shows that the pyrophosphatase is still markedly active at the normal *p*H of milk. This enzyme, if present in milk, would presumably decompose cocarboxylase to the mono-

phosphate ester of vitamin B_1. A phosphomonoesterase active at the same *p*H would then be necessary to complete the process.

(*c*) *The cocarboxylase-splitting enzymes of milk.*

The observations on colostral milk definitely suggest that a parallelism exists in milk between alkaline phosphomonoesterase and the enzymes capable of complete dephosphorylation of cocarboxylase at the normal *p*H of milk. Incubation of milk of different phosphomonoesterase content, with or without addition of solutions of cocarboxylase, has yielded evidence to the same effect (Table 13). In the first instance we have been able to show that late lactation milk, very rich in phosphomonoesterase and containing little or no natural cocarboxylase, can rapidly dephosphorylate added cocarboxylase. In early lactation milk, however, which is low in phosphomonoesterase and rich in cocarboxylase, the natural cocarboxylase is not dephosphorylated on incubation at the natural *p*H though it is easily broken down by takaphosphatase at *p*H 4. Full lactation milk of moderate phosphomonoesterase activity occupies an intermediate position. The incubation of such milk, with or without added cocarboxylase, results in partial dephosphorylation of the added or natural aneurin pyrophosphate. This effect in late lactation milks is quantitative and so rapid that even after 1 hr. at room temperature some of the added cocarboxylase is already split.

Table 13. *Incubation of early and late lactation milks separately and mixed, with and without added cocarboxylase*

			Vitamin B_1 in μg./100 ml.			
				After incubation at 37° C.		
Type of milk		Phosphomonoesterase in King and Armstrong units	"Free"	At *p*H 4	At natural *p*H	With takaphosphatase at *p*H 4
Early lactation	1	17·0	9·1	9·9	10·2	52·5
	2	17·9	13·5	12·3	13·2	50·4
	3	26·9	9·3	8·4	7·8	81·3
Full lactation bulk milk	Alone	>137·0	25·4	—	29·1	31·5
	+21 μg. vitamin B_1 as cocarboxylase		25·4	28·5	37·5	49·5
Late lactation milk I	Alone	270·0	29·1	—	25·4	27·6
	+13·2 μg. vitamin B_1 as cocarboxylase		27·9	30·0	38·4	42·0
Late lactation milk II	Alone	365·0	30·0	30·6	29·6	31·1
	+52·8 μg. vitamin B_1 as cocarboxylase		34·2*	32·8	83·3	84·0
Late lactation milk III	Alone	321·5	35·3	34·1	33·3	35·9
	+26·8 g. vitamin B_1 as cocarboxylase		37·1	33·8	59·2	60·8
Mixed early and late lactation milk (1:1)		165·4	31·3	33·9	40·8	52·0
Early lactation milk		41·8	34·2	—	—	66·9
Late lactation milk		365·0	35·4	—	—	34·1
Mixed early and late lactation milk (1:1)		168·9	31·3	31·5	40·0	49·4
Mixed early and late lactation milk (1:1) +33·0 μg. vitamin B_1 as cocarboxylase			35·4	35·9	58·3	85·4

* Measured 1 hr. after mixing; this went up to 49·2 after 4 hr. at room temperature.

Milks of high phosphomonoesterase activity dephosphorylate not only added but also the natural cocarboxylase of milk. This has been proved by mixing a late lactation milk, high in phosphomonoesterase, with an equal volume of milk from a recently calved cow, high in cocarboxylase. This conversion, as can be seen from Table 13, was not quantitative but amounted to some 50 %. This was not due to the lower enzyme titre of the mixed milk, since in addition to the natural cocarboxylase such milk could deal with added cocarboxylase, though again only to the extent of 50 %.

(*d*) "*Free*" *and phosphorylated vitamin* B_1 *in the milk of the goat.*

The phosphomonoesterase content of goat's milk is very low (18) and it was of interest to see whether the same close relation exists in this animal as in the cow, between this substance and the "free" vitamin B_1 of milk.

The milk of five goats was examined by the usual technique for vitamin B_1. One of the goats was in very advanced lactation and was going dry. The phosphatase content of her milk was abnormally high. The results are given in Table 14.

Table 14. *The phosphomonoesterase content and the "free" and total vitamin* B_1 *of goat's milk*

Goat no.	Days in lactation	Phospho-monoesterase in King and Armstrong units	Vitamin B_1 in μg./100 ml.	
			"Free"	Total
1	478	38·6	10·8	57·7
	483	130·4*	22·8	56·4
2	213	—	6·1	57·8
	218	6·2	4·2	57·2
3	192	8·5	7·2	57·3
	197	9·5	7·8	47·4
4	188	5·8	7·8	66·6
	193	7·7	7·5	59·1
5	146	6·6	5·1	67·8
	151	11·8	6·3	49·5

* Almost dry.

It will be seen that goat's milk is some 50 % richer in vitamin B_1 than the milk of cows in full lactation but that the phosphatase is very low. In this respect full lactation goat's milk closely resembles the milk from cows in early lactation. The similarity extends further to the low "free" vitamin B_1 and high cocarboxylase characterized by strong fluorescence in the aqueous layer in the thiochrome test. Towards the close of lactation the milk of goat no. 1 showed a marked rise in the phosphatase titre and a corresponding rise in the "free" vitamin B_1. It therefore appears that, as for cow's milk, the alkaline phosphomonoesterase of goat's milk is a guide to the concentration of enzymes capable of splitting cocarboxylase.

(*e*) *Effect of frequent milkings on the partition of vitamin B_1 in milk.*

The rapidity with which a milk of high phosphatase titre dephosphorylates added cocarboxylase suggests that a similar reaction may be taking place in the udder itself and that cocarboxylase measured in milk obtained by the usual twice daily milkings may differ in amount from that present in milk immediately after secretion. Two cows were milked at hourly and half-hourly intervals after the morning milking and the usual tests were carried out immediately after each milking. The results are given in Table 15.

Table 15. *Effect of frequent milkings on the partition of vitamin B_1 in milk. L, Lottie* 7*; F, Flora* 43

Interval since last milking hr.		Vitamin B_1 in μg./100 ml. "Free"		Total		"Free" as % of total		Phosphomonoesterase in King and Armstrong units		Milk yield ml.	
L	F	L	F	L	F	L	F	L	F	L	F
15	15	10·1	28·3	26·5	34·5	38·1	82·0	52·7	160·2	6580	4540
1	1	3·5	23·3	22·4	29·8	15·6	78·2	—	230·0	370	150
1	1	7·9	19·2	10·0	24·5	79·0	78·4	—	—	70	45
1	1	8·4	22·2	24·0	35·3	35·0	61·2	79·6	220·6	270	567
—	½	—	23·8	—	35·0	—	68·0	—	246·8	—	125
1	½	8·4	14·6	23·2	—	37·8	—	—	—	150	12
1	1	9·7	25·4	23·3	38·5	41·6	66·0	—	224·6	793	163
1	1	11·7	25·4	27·2	37·3	43·0	68·1	58·4	—	680	567
1	1	11·9	23·3	30·6	37·3	38·9	62·5	—	—	680	227
1	1½	9·2	25·0	28·9	38·9	31·8	64·3	—	206·8	454	227

Lottie 7 had a relatively low milk phosphatase and her initial "free" vitamin B_1 was only a third of the total. There was a marked further drop at the next milking and a sharp recovery at the following; thereafter the level rose to reach and surpass in the last four tests the initial value. The total vitamin B_1 was also reduced. The ratio of "free" to total, with the exception of the second test where it fell markedly, and of the third where, owing to the anomalous value for total vitamin B_1, it was very high, remained practically unaltered throughout the test.

The milk of the second cow had a much higher phosphatase titre and her initial "free" vitamin B_1 amounted to 80 % of the total. Here the frequent milkings brought about a noticeable fall in the "free" vitamin B_1. At first the total vitamin B_1 also declined, but it rose later above the initial value and, as a result, the "free" fraction accounted for only about 65 % of the total. It is interesting to note that the smallest sample of milk, 12 ml. obtained after a ½ hr. interval, contained only half the initial "free" vitamin B_1. Unfortunately, there was not enough milk for an assay of total vitamin B_1 and the significance of the fall cannot be assessed. The second lowest figure for "free" vitamin B_1, also from a small sample, was accompanied by a low total vitamin B_1 and the ratio was unchanged.

It will be noticed that for both cows the phosphatase readings increased

well above the initial values and then fell again. It seems likely that the increase was connected with the frequent handling of the udder during milking.

While the result of these experiments is by no means decisive, it suggests that in milks of high phosphatase activity enzymic dephosphorylation of cocarboxylase may take place in the udder.

(*f*) *The source of the vitamin B_1 of milk.*

Cocarboxylase is found in most milks and it seems possible that the substance may be originally present even in those in which it cannot be detected some time after the actual secretion of the milk has taken place. The rapid disappearance from such milks of added cocarboxylase supports this view. While some of the milk cocarboxylase is bound, a part of it in early milks is present in a freely diffusible form (cf. p. 151) and the question of the origin of these substances is therefore of importance. It is clear from the work of Sinclair that they are not derived from the blood by simple filtration, as all the blood cocarboxylase is found within the blood cells and is, moreover, in a combined form (Goodhart & Sinclair[19]), while the vitamin B_1 of normal cerebrospinal fluid, a simple filtrate, is free and unphosphorylated (Sinclair[20]). Fixed tissue cells contain a large part of their vitamin B_1 as cocarboxylase[21,22] and it seems very likely that milk cocarboxylase arises directly from the secretory cells of the mammary gland. It is very probable that these cells liberate at the same time enzymes capable of dephosphorylating cocarboxylase; it is definitely known that milk phosphatase arises in this way[14] and there is little doubt that the assay of this alkaline monophosphoesterase can serve as a very good guide to the concentration of the enzymes involved in the splitting up of cocarboxylase at the natural *p*H of milk.

Probably a part at least of the unesterified vitamin B_1 present in milk is formed by enzymic hydrolysis of cocarboxylase after secretion. Whether both the vitamin B_1 and the cocarboxylase of milk are solely derived from the mammary cells or whether blood plasma contributes its quota of diffusible vitamin B_1 is at present a matter of speculation. In this respect, two observations are of significance. In the first place the concentration of diffusible vitamin B_1 in plasma is very low, about 1 μg./100 ml.[20,23], the remaining vitamin B_1 being firmly bound to protein[19] and therefore not diffusible. The well-known ability of the mammary gland to concentrate enormously in the course of secretion certain diffusible blood constituents may well compensate for this deficiency. It may be that one method of concentration of vitamin B_1 from the circulating plasma is adsorption on the proteins of the secreting cells and discharge of these proteins into the alveolar spaces in due course. Of much greater importance is the fact that in colostrum and in early milk over 80 % of the total vitamin B_1 may be in the form of free and bound cocarboxylase and hence be derived not from blood plasma but from formed elements. Moreover, a not insignificant proportion of the unphosphorylated vitamin B_1 in such milk is combined in larger complexes and is not diffusible. It is most

unlikely that such combinations are formed in milk after it has been secreted, but it is easy to visualize their appearance in the course of breakdown of secretory cells. Table 7 shows that more than 90 % of the vitamin B_1 and cocarboxylase of a colostrum is present in the non-diffusible form, and in this case at any rate the blood plasma apparently contributes less than one-tenth of the total vitamin B_1 present. Deeper insight into the origin of the vitamin B_1 of milk might be gained by simultaneous study of milk, arterial blood and blood from the mammary vein by the method of Graham *et al.* (24).

(2) *Seasonal changes*

Two series of measurements on pooled Shorthorn milk, covering both stall and pasture feeding, have been carried out.

(*a*) *First experiment.*

In the first (1938) series only the "free" vitamin B_1 was measured. The bulk milk of six Shorthorn cows of which five were in full and one in early lactation was examined over a period extending from 9 March to 3 June 1938.

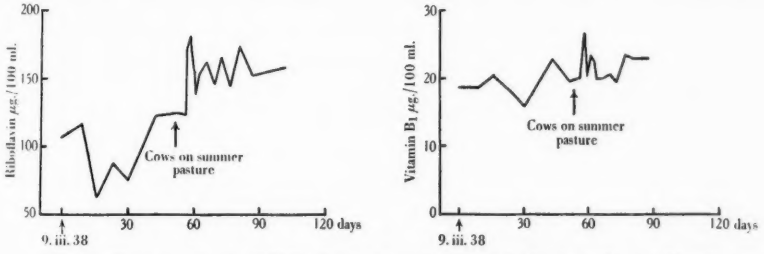

Fig. 2. First experiment (1938). Seasonal variation of riboflavin (left) and "free" vitamin B_1 (right) in the bulk milk of six Shorthorn cows.

The winter ration consisted of 10 lb. hay, 50 lb. mangels and 1½ lb. maize, with the addition of a concentrate ration fed at the rate of 4 lb./gal. of milk and consisting of decorticated ground nut meal, wheat offals, flaked maize and minerals. Till 1 May the cows had access to bare pasture. During the last fortnight of March there was a definite spring growth.

The cows went out to summer grazing on 1 May. The quality of the pasture was, however, very much below the average for the south of England, owing to abnormally dry conditions. For this reason the cows were continued till 12 May on the concentrate ration and received also 5 lb. of hay, mangels being omitted. After 12 May, the feeding of hay was discontinued and the concentrate ration was gradually reduced as more grass became available. The supplement of 1½ lb. of flaked maize was continued throughout.

Vitamin B_1 estimations were done on samples of pooled morning and evening milk blended in the proportion of 6:4. Samples were taken at weekly intervals except when cows went out to pasture, when daily samplings were made. The results are presented in Fig. 2.

(*b*) *Second experiment.*

After the technique for the estimation of the total vitamin B_1 had been developed a second experiment was carried out covering the period between 8 March and 1 December 1939. Two groups of three Shorthorn cows were used; one group was kept indoors on a winter ration till 10 July, the other went out to pasture in spring in the usual manner. Full details of the feeding and

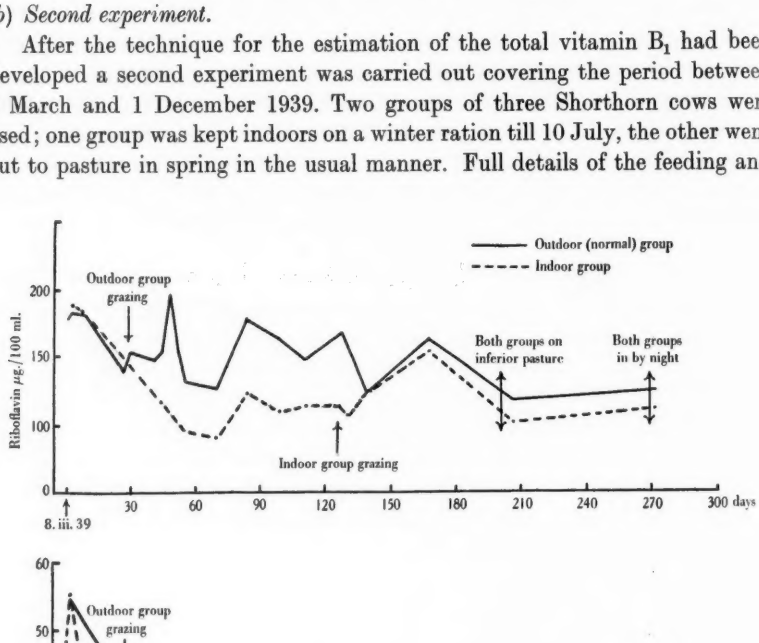

Fig. 3. Second experiment (1939). Seasonal variation of riboflavin (top) and "free" and total vitamin B_1 in the milk of two groups of Shorthorn cows.

——— Indoor group. Total vitamin B_1. – – – – Outdoor group. Total vitamin B_1.
—·— Indoor group. "Free" vitamin B_1. . . . . Outdoor group. "Free" vitamin B_1.

management of these cows until 10 July are given in a paper by Bartlett *et al.* (25). On 10 July the indoor group was put out to grass alongside the outdoor group. In October, as the available grass diminished, the feeding of hay and of cabbage (later replaced by kale) was started. After 27 November all cows were kept in at night. Tests were carried out as before on blended morning and evening samples of milk, both "free" and total vitamin B_1 being measured. The results are given in Fig. 3.

(*c*) *Results.*

It has been shown earlier in this paper that the stage of lactation exerts a marked effect on the vitamin B_1 content of milk and in considering seasonal changes this effect must be taken into account. In this respect, the indoor group of cows in the second experiment served as a control for the normal or outdoor group. At the beginning, both groups were in early lactation and the total vitamin B_1 content of the milk was high. It declined slowly for both groups from an initial value of 45–55 μg./100 ml. to a steady level of from 30 to 36 μg./100 ml., while in the same period the "free" vitamin B_1 rose from about 22 to 30 μg./100 ml. These changes are similar to those observed with progressing lactation in individual cows (p. 156 and Fig. 1). Neither the change to grass of one group nor the later change in the diet of the other group had any detectable effect. A similar picture is given by the results of the first experiment. There the cows were in later lactation and the change in the "free" vitamin B_1 was not so marked, though the upward trend towards the end of the experiment was apparent. With both groups the experiment was stopped when some of the cows went dry. The figures for "free" vitamin B_1 agree well with those quoted by Wiegand (13). Our results show that the vitamin B_1 content of milk is independent of the nutrition of the cow. This is in agreement with the results of other investigators whose work has been reviewed by Kon (26).

B. Riboflavin

(1) *Stage of lactation*

Riboflavin measurements were carried out on the same milks as were used for the estimation of vitamin B_1. The riboflavin was estimated fluorimetrically by the method of Emmerie (27) in the outfit described by Henry *et al.* (7). The method of Emmerie involves heating in methanol solution, but measurements were also carried out in which the heating was omitted. In this way a differentiation was obtained between "free" riboflavin and that bound to protein (27). The results for the seven cows treated individually (cf. p. 155) are given in graphic form in Fig. 4. Very high values were found for samples of colostrum; these declined rapidly to normal milk values. The concentration of riboflavin in the first and second colostrum was some 3–4 times greater than in later milk. This is in agreement with earlier findings of Kramer *et al.* (28).

(2) *Seasonal changes*

(*a*) *First experiment.*

The same cows were used as described on p. 163. In this test riboflavin was estimated by the method of Whitnah *et al.* (29). The results are plotted in Fig. 2 and show that a definite rise in the riboflavin of milk is effected by pasture.

(*b*) *Second experiment.*

The two groups of cows used in this experiment were those described on p. 164. The method of Emmerie (27) was used for the riboflavin determinations.

The results are given in Fig. 3. Here again, the effect of pasture can be clearly seen. The riboflavin content of the milk of the outdoor group of cows fluctuated around 150 μg./100 ml. during the pasture season, while that of the indoor group was lower during the same period, about 110 μg./100 ml. This value rose

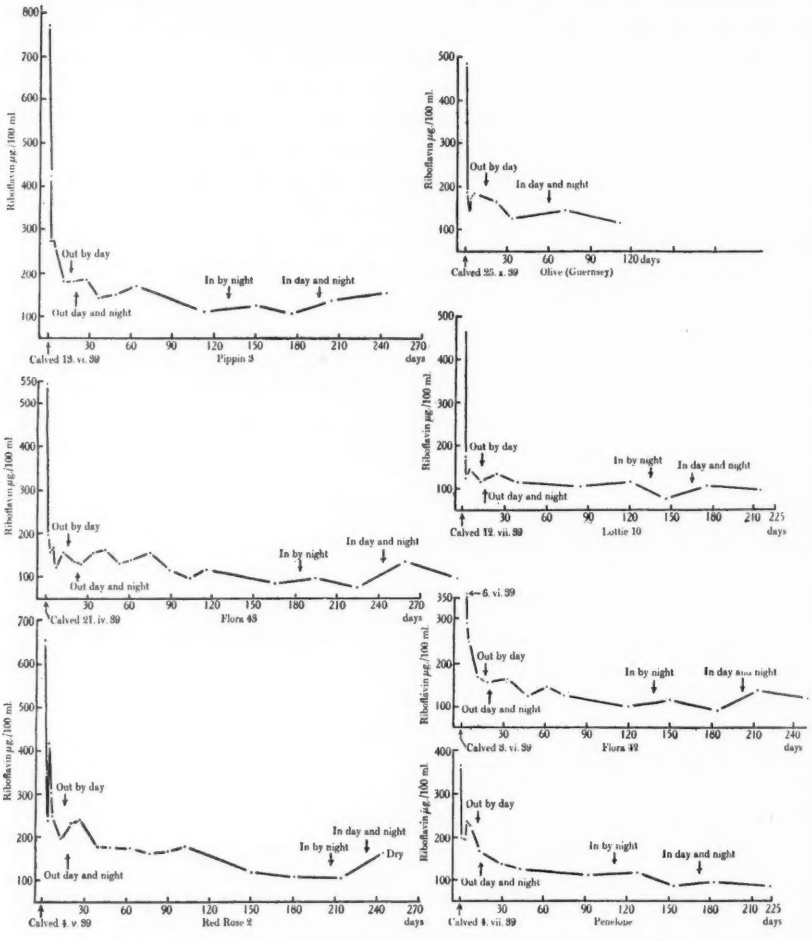

Fig. 4. Riboflavin content of the milk of six Shorthorn cows and one Guernsey cow in the course of lactation.

when the indoor group went out to grass in July, and the curves for the two groups joined at this stage. They then declined together as pasture deteriorated. Similar seasonal differences in the riboflavin level of milk will be found in the paper of Houston *et al.* (30). This is in agreement with the observations of Virtanen & Holmberg (31) and Hand & Sharp (32). It should be pointed out,

however, that Kramer *et al.* (33) doubt, on the basis of biological tests, whether the riboflavin content of milk is much affected by the diet of the cow.

In all our measurements we found that about 10 % of the total riboflavin was present in combination with protein. As the fluctuations in this ratio were slight, only total figures are given in Figs. 2–4.

PART IV. COMPARISON OF CHEMICAL AND BIOLOGICAL METHODS OF ESTIMATION OF VITAMIN B_1

A. INTRODUCTION

To be accepted with confidence a chemical method for the estimation of a biologically active substance should agree with results of biological tests. For this reason an attempt was made to check by animal experiments the steps of development of the Jansen thiochrome test for vitamin B_1 as applied to milk, which has been described in the preceding pages. These comparisons of fluorimetric and biological assay, which extended over two years, may be divided into two stages. In the first the Jansen test measured, as we know now, only the "free" vitamin B_1 and the discrepancy which was in effect found between the two methods was only to be expected. In the later stage, all forms of vitamin B_1 present in milk were, to the best of our knowledge, measured by the modified chemical technique. The differences which even then were noted in some cases may probably be attributed to certain difficulties in the biological technique which will be discussed later.

Seven series of biological tests were carried out. In these not only raw milk was tested but also spray- and roller-dried, evaporated and sterilized milk. A change in the International Standard for vitamin B_1 took place while these experiments were in progress. Through the courtesy of the Medical Research Council, we were given access to the new crystalline vitamin B_1 preparation before the official date of issue. This was used from the beginning of the second experiment. The general technique of the biological tests was as described by Henry *et al.* (7). Any departures from this technique will be noted below.

B. EXPERIMENTAL

(1) *First experiment* (*October* 1937)

Roller-dried, spray-dried and evaporated milk prepared from one bulk of raw milk and a raw milk of different origin were used in the tests. The results of the biological part of this test have already been reported (Henry *et al.* (7)). The basal diet used (diet 1) is given in Table 16. For comparison with fluorimetric tests the average gains in weight of the experimental animals and the vitamin B_1 potency of the milks calculated as described on p. 175 are given in Table 17. Fluorimetric measurements were made as described on p. 146, i.e. only "free" vitamin B_1 was estimated. The raw milk was tested daily, the dried and evaporated samples once a week. The results are given in Table 17.

Table 16. *Composition of experimental vitamin B_1-deficient diets*

Components	Diet 1	Diet 2	Diet 3*	Diet 4*	Diet 5*
Casein (New Zealand lactic casein washed)†	19·2	24·0	—	—	—
Casein (Glaxo A/E)	—	—	20·0	16·9	17·9
Dextrinized potato starch	53·6	67·0	—	—	—
Rice starch	—	—	60·0	52·2	55·4
Ground-nut oil	4·0	5·0	12·0	6·7	7·2
Lard	—	—	3·0	—	—
Salts (Steenbock's 40)	3·2	4·0	5·0	4·2	4·5
Autoclaved yeast	20·0	—	—	—	—
Autoclaved whey	—	—	—	20·0	—
Autoclaved pig's liver	—	—	—	—	15·0

* These diets were mixed with water and steamed as described by Edgar *et al.*(34).

† The casein is washed at the isoelectric point six times with water acidulated with HCl.

N.B. All animals received two drops of cod-liver oil daily separately from the diet.

These show that the agreement between the biological and the chemical method of estimation of vitamin B_1 was unsatisfactory since, when extrapolated results were disregarded, the fluorimetric assay yielded values only one-half to two-thirds as high as those given by the animal test.

(2) *Second experiment* (*June* 1938)

A crystalline preparation of vitamin B_1 identical with the Second International Standard vitamin B_1, officially issued in November 1938, was used in this experiment (cf. p. 167). For dosing, a stock solution containing 1000 μg./ml. was prepared in 25 % aqueous ethyl alcohol in *N*/500 HCl. A subsidiary dilution of 50 μg./ml. in *N*/500 HCl was made every second day and from this the solution for animal dosage (containing 15 μg./ml.) was made up daily. All solutions were kept at 0° C. The standard was fed at levels of 0·25, 0·5, 1·0, 2·0 and 4·0 I.U. daily, 1 I.U. being by definition, equal to 3 μg. of vitamin B_1. Diet 1 (Table 16) was again used. The animals, when "run out", were placed in individual cages. There were five bucks and five does on each treatment. Spray-dried full cream milk and evaporated full-cream milk obtained from one bulk of raw milk were reconstituted each day to the same total solids content as the original milk and fed at levels of 6 and 12 ml. daily and 8 and 16 ml. daily respectively. A fresh tin of evaporated milk was opened each day. One batch of sterilized milk was given at the rate of 6 and 12 ml. daily, a fresh bottle being opened for each sampling. Finally, raw milk from the Institute's herd was fed at levels of 4 and 8 ml. daily. Fluorimetric tests (p. 146) were carried out once a week on the first three milks and daily on the raw. The results are given in Table 18, from which it is again evident that a fluorimetric test measuring only "free" vitamin B_1 does not agree with the biological tests. The nearest agreement was with sterilized milk. This is understandable in view of the later findings of Houston *et al.* (30) that in the sterilization of milk the combined forms of vitamin B_1 are more readily destroyed than the "free".

Table 17, Exp. 1. *Mean gains in weight for standard and milks, 6 ♂♂ and 6 ♀♀ in each group. Vitamin B_1 potency of the milks estimated biologically and fluorimetrically*

		Gain in weight (g.)				Vitamin B_1 potency of milks					
						Biological estimations				Fluorimetric estimations	
		Males		Females		Males		Females			
		Gain	S.E.M.*	Gain	S.E.M.*	I.U./100 ml. milk	C.V.† %	I.U./100 ml. milk	C.V.† %	I.U./100 ml. milk	S.E.M.*
Standard‡	1·0 I.U.	39·5	±5·72	23·3	±1·46	—	—	—	—	—	—
	1·5 I.U.	51·3	±4·88	34·2	±1·25	—	—	—	—	—	—
	2·0 I.U.	51·2	±3·02	41·9	±1·40	—	—	—	—	—	—
	3·0 I.U.	60·7	±4·12	52·8	±1·76	—	—	—	—	—	—
Roller-dried milk I	8 ml.	19·5§	±5·88	33·3	±3·44	3·8‖	34·0	18·1	11·7	7·2	±0·28
	12 ml.	48·4	±5·02	47·5	±2·14	12·7	30·6	20·6	8·5		
Spray-dried milk I	8 ml.	19·0§	±7·06	29·7	±3·44	3·7‖	41·8	15·9	11·7	10·5	±0·20
	12 ml.	50·0	±4·45	47·3	±4·23	13·9	27·8	20·4	16·0		
Evaporated milk II	8 ml.	11·3§	±6·64	10·2	±2·01	2·4‖	39·8	7·7‖	8·1	6·7	±0·15
	12 ml.	18·6	±5·53	27·5	±2·47	2·4‖	33·9	9·8	9·7		
Raw milk	8 ml.	31·3	±2·86	25·7	±0·89	7·4‖	20·6	13·6	4·4	9·5	±0·30

Male line equation: mean gain (g.) = 41·5 log dose + 40·8. Female line equation: mean gain (g.) = 61·8 log dose + 23·3.

* S.E.M. = standard error of mean. † C.V. = coefficient of variation.

‡ Acid clay standard V.B_1. 11. § One animal lost weight. ‖ By extrapolation.

Table 18, Exp. 2. *Mean gains in weight for standard and milks. 5 ♂♂ and 5 ♀♀ in each group. Vitamin B_1 potency of the milks estimated biologically and fluorimetrically*

		Gain in weight (g.)				Vitamin B_1 potency of milks					
						Biological estimations				Fluorimetric estimations	
		Males		Females		Males		Females			
		Gain	S.E.M.*	Gain	S.E.M.*	I.U./100 ml. milk	C.V.† %	I.U./100 ml. milk	C.V.† %	I.U./100 ml. milk	S.E.M.*
Standard‡	0·25 I.U.**	—	—	−5·0	±8·73	—	—	—	—	—	—
	0·5 I.U.	17·8	±2·71	21·0§	±6·43	—	—	—	—	—	—
	1·0 I.U.	28·6	±2·38	31·6	±3·57	—	—	—	—	—	—
	2·0 I.U.	49·4	±5·40	47·4	±3·23	—	—	—	—	—	—
	4·0 I.U.	50·6	±8·22	42·0	±2·76	—	—	—	—	—	—
Raw milk	4 ml.	14·0	±6·52	17·2	±3·71	(*a*) 11·25¶ (*b*) 11·50¶	40·2	9·00¶ 11·75¶	40·4	7·51	±0·368
	8 ml.	44·8	±3·73	34·2	±5·63	(*a*) 25·12 (*b*) 25·62	28·0	17·50 11·62	49·5		
Sterilized milk I	6 ml.	9·4‖	±11·39	5·2§	±5·65	(*a*) 5·67¶ (*b*) 6·17¶	65·1	2·33¶ 5·67¶	62·2	5·27	±0·103
	12 ml.	29·0	±5·72	35·4	±3·78	(*a*) 7·91 (*b*) 7·66	33·4	12·90 8·33	36·8		
Spray-dried milk II	6 ml.	28·8	±1·36	39·6	±3·74	(*a*) 16·00 (*b*) 15·18	15·8	36·00 22·20	38·6	6·10	±0·361
	12 ml.	64·2	±5·92	54·4	±1·17	(*a*) 51·25¶ (*b*) 85·90¶	47·4	59·0¶ ?	43·3		
Evaporated milk III	8 ml.	20·8	±5·67	29·8	±6·31	(*a*) 7·75 (*b*) 7·75	34·3	12·38 9·50	54·0	3·13	±0·114
	16 ml.	53·0	±9·69	50·4	±5·45	(*a*) 21·30¶ (*b*) 24·50¶	57·8	32·1¶ ?	57·1		

(*a*) Straight-line equation: males, mean gain (g.) = 43·4 log dose + 29·9; females, mean gain (g.) = 28·8 log dose + 29·9.

(*b*) Parabola equation: males, mean gain (g.) = 30·6 + 44·4 log dose − 11·05 (log dose)²; females, mean gain (g.) = 35·5 + 41·3 log dose − 48·00 (log dose)².

* S.E.M. = standard error of mean. † C.V. = coefficient of variation.

‡ Crystalline vitamin B_1 identical with the Second International Standard for vitamin B_1.

** All but one male died before the end of the experiment and four out of five females lost weight. Results not used in calculation of line equations

§ One animal lost weight. ‖ Three animals lost weight. ¶ By extrapolation.

(3) *Third experiment* (*January* 1939)

In this experiment the basal diet used in the biological tests was changed and the fluorimetric test was modified to estimate not only the "free" but the total vitamin B_1 of milk (cf. p. 148). In the two previous experiments some delay was experienced in getting the animals "run out" on the basal diet. After consultation with Dr Chick and Dr Macrae of the Lister Institute it was decided to use the Lister vitamin B_1-deficient diet, the composition of which is given in Table 16 (diet 3). The method of preparation is given by Edgar *et al.* (34). The animals were weaned on to our vitamin B complex deficient diet (diet 2, Table 16) and transferred within a few days to the new diet. After a week they were placed in individual cages and given 1 ml. daily of an autoclaved aqueous yeast extract equivalent to 1 g. of dry yeast. The yeast extract was prepared as follows: 5 kg. of fresh pressed brewer's yeast were boiled for 10 min. with 10 l. of tap water containing 1 ml. of glacial acetic acid, filtered through Buchner funnels and washed with water. The combined extracts were evaporated in an open basin over a small flame to about 1 l. and then autoclaved for 5 hr. at 120° C., centrifuged and the supernatant fluid made to volume.

The rats became "run out" on this diet in 3–4 weeks. Groups of ten were placed on each treatment. Spray-dried and evaporated skim milk prepared from one bulk of raw milk and the spray-dried full-cream milk used in the first experiment were both tested. The milks were fed at levels calculated from fluorimetric tests to supply about 1 and 2 I.U. daily. This amounted for evaporated separated milk to 4·5 and 9·0 g., for spray-dried separated milk to 0·5 and 1·0 g. and for spray-dried full-cream milk to 1·0 and 2·0 g. daily respectively. The standard was given at levels of 1, 2 and 4 I.U. daily. Fluorimetric estimations were carried out once a week. For feeding, the two spray-dried samples were each day dissolved in five times their weight of water and measured out, while the evaporated milk was weighed out and fed in glass dishes under hoods (35), a fresh tin being opened daily. The results of this experiment are shown in Table 19. The presumptive levels of feeding of the spray-dried separated milk were lower than planned, i.e. 0·83 and 1·67 I.U., and the gain in weight of the animals receiving the lower level was so much below that given by the 1 I.U. level of the standard that a calculation of potency would involve unjustified extrapolation. The two highest levels of standard produced very similar growth responses and as a result the calculation of potency of the higher levels of the two other milks was made uncertain. With these reservations, the agreement between the biological and fluorimetric methods was much improved in comparison with Exps. 1 and 2. However, the evaporated milk, as in the previous experiments, still gave a systematically higher potency by the biological method while the spray-dried milks gave a higher potency by the fluorimetric method. A further experiment was therefore carried out.

Table 19, Exp. 3. *Mean gains in weight for standard and milks. The number of animals used is given in brackets after the gains. Vitamin B_1 potency of the milks estimated biologically and fluorimetrically*

		Gain in weight (g.)				Vitamin B_1 potency of milks					
						Biological estimations				Fluorimetric estimations	
		Males		Females		Males		Females			
		Gain	S.E.M.*	Gain	S.E.M.*	I.U./g. milk	C.V.† %	I.U./g. milk	C.V.† %	I.U./g. milk	S.E.M.*
Standard‡	1·0 I.U.	30·6 (5)	±5·56	34·8 (5)	±2·84	—	—	—	—	—	—
	2·0 I.U.	70·6 (5)	±1·69	59·5 (4)	±1·76	—	—	—	—	—	—
	4·0 I.U.	79·7 (6)	±4·63	55·5 (4)	±4·50	—	—	—	—	—	—
Spray-dried skim milk	0·5 g.	6·3 (4)‖	±7·30	18·7 (6)§	±7·99	(*a*) 0·64¶ (*b*) 1·52	33·3	0·700¶ 1·56¶	47·3	1·67	±0·038
	1·0 g.	63·0 (6)	±5·82	50·3 (4)	±4·15	(*a*) 1·73 (*b*) 1·67	18·9	1·58 1·39	21·9		
Evaporated skim milk	4·5 g.	58·8 (5)	±3·64	47·6 (5)	±2·98	(*a*) 0·340 (*b*) 0·340	13·6	0·309 0·289	17·5		
	9·0 g.	78·0 (6)	±4·63	68·8 (4)	±3·99	(*a*) 0·300 (*b*) {0·296 {0·503	16·1	0·423¶ ?	23·1	0·234	±0·003
Spray-dried milk I	1·0 g.	29·8 (5)§	±10·36	33·2 (5)	±3·75	(*a*) 0·644¶ (*b*) 0·991¶	34·8	0·700¶ 0·970¶	25·5	0·92	±0·187
	2·0 g.	56·0 (6)	±4·86	49·5 (4)	±5·39	(*a*) 0·701 (*b*) 0·725	16·9	0·760 0·685	27·4		

(*a*) Straight-line equation: males, mean gain (g.) = 44·6 + 77·5 log dose; females, mean gain (g.) = 40·6 + 48·5 log dose.
(*b*) Parabola equation: males, mean gain (g.) = 30·6 + 184·0 log dose − 170·0 (log dose)²; females, mean gain (g.) = 34·8 + 129·9 log dose − 158·4 (log dose)².

* S.E.M. = standard error of mean. † C.V. = coefficient of variation. ‡ Second International Standard for vitamin B_1.
§ One animal lost weight. ‖ Two animals lost weight. ¶ By extrapolation.

(4) *Fourth experiment* (*April* 1939)

Animals were placed at weaning, or as soon as they weighed 40 g., on diet 3 (Table 16), and were individually caged a week later and given, as in the previous experiment, 1 ml. daily of autoclaved yeast extract. As the rats became "run out" they were placed on the various treatments in groups of twelve (6♂♂ and 6♀♀). The standard was fed at levels of 0·75, 1·5 and 3·0 I.U. daily. The roller-dried and evaporated milks used in the first experiment and a sample of sterilized milk were used in this experiment and were fed at presumptive levels of approximately 0·75 and 1·5 I.U. daily. The corresponding amounts were for roller-dried milk 1 and 2 g., for evaporated milk 5 and 10 g., for sterilized milk 10 and 20 ml. The roller-dried milk was reconstituted daily in such a way that 1 g. was contained in 7·5 ml. of the solution. The evaporated milk was weighed out as in the previous experiment. Fluorimetric tests were done once a week. The results are given in Table 20. The agreement was again bad for evaporated milk. For the other two it was satisfactory only at the lower levels of feeding. Further experiments in which the basal diet was altered were therefore carried out.

(5) *Fifth experiment* (*April* 1939)

There were indications from the first three experiments that the calculated potency of a given sample of milk varied with the level fed, the higher level giving a greater potency; in other words, the weight dosage curve rose more steeply for the milks than for the crystalline vitamin B_1. Such a result suggested that the basal diet was deficient in something else besides vitamin B_1 and that milk made good this deficiency. Schlutz & Knott (36) had suggested the use of autoclaved whey in preference to autoclaved yeast as a source of the heat stable vitamin B complex in vitamin B_1 studies, an opinion shared by Daniels (37). As one might expect autoclaved whey to contain the water soluble thermostable principles found in milk, a new diet containing autoclaved whey, i.e. diet 4, Table 16, was tried out. For autoclaving the whey was mixed with an equal weight of water and heated to 120° C. for 2 hr. Male rats were placed on diet 4 at weaning, and were ready for tests within 4 weeks. A preliminary trial was then carried out simultaneously with the fourth experiment. One group of eight males was given 1·5 I.U. of standard and a similar group received the same presumptive level of vitamin B_1 contained in 2 g. of roller-dried milk. The mean gain of the rats receiving the standard was $43{\cdot}5 \pm 5{\cdot}1$ g. and that of the milk rats $56{\cdot}4 \pm 4{\cdot}8$ g. These gains are lower than the corresponding figures obtained in Exp. 4, but there was still evidence that the biological method was giving higher estimates of potency than the fluorimetric.

(6) *Sixth experiment* (*June* 1939)

That the autoclaved whey diet was no more satisfactory for our purpose than the autoclaved yeast was confirmed by a further paper by Schlutz & Knott (38), who advised the use of autoclaved liver. This was also borne out by

Table 20, Exp. 4. *Mean gains in weight for standard and milks. 6 ♂♂ and 6 ♀♀ in each group. Vitamin B_1 potency of the milks estimated biologically and fluorimetrically*

		Gain in weight (g.)				Vitamin B_1 potency of milks					
						Biological estimations					
		Males		Females		Males		Females		Fluorimetric estimations	
		Gain	S.E.M.*	Gain	S.E.M.*	I.U./g. milk	C.V.† %	I.U./g. milk	C.V.† %	I.U./g. milk	S.E.M.*
Standard‡	0·75 I.U.	26·2	±4·36	28·0	±2·85	—	—	—	—	—	—
	1·5 I.U.	57·3	±3·43	46·5	±1·29	—	—	—	—	—	—
	3·0 I.U.	79·5	±1·53	60·8	±3·33	—	—	—	—	—	—
Evaporated milk II	5 g.	42·7	±2·86	37·5	±3·00	0·22	11·1	0·19	15·7	0·15	±0·007
	10 g.	76·3	±5·21	63·5	±1·83	0·29	19·0	0·31	19·5		
Roller-dried milk I	1 g.	44·5	±3·46	35·3	±2·43	1·11	11·9	0·97	15·5	0·79	±0·015
	2 g.	72·5	±2·69	64·0	±3·03	1·23	12·9	1·62	22·3		
						I.U./100 ml.		I.U./100 ml.		I.U./100 ml.	
Sterilized milk II	10 ml.	34·5	±2·82	23·5	±5·25	8·70	13·7	5·91§	30·3	7·55	±0·106
	20 ml.	66·0	±2·56	56·2	±2·94	10·30	10·6	11·40	16·9		

Male-line equation: mean gain (g.) = 84·4 log dose + 39·5; female-line equation: mean gain (g.) = 55·6 log dose + 36·2.

* S.E.M. = standard error of mean.
† C.V. = coefficient of variation.
‡ Second International Standard for vitamin B_1.
§ By extrapolation.

Daniels(39). A new diet containing pig's liver autoclaved by the method of Schlutz & Knott(38) was therefore prepared (diet 5, Table 16). The results of tests in which two groups of animals received respectively 0·75 and 1·5 I.U. of the standard and a third 6 g. of evaporated milk equivalent to a presumptive level of 0·85 I.U. of vitamin B_1 are given in Table 21. This time the agreement was excellent.

(7) *Seventh experiment* (*October* 1939)

An experiment on a larger scale was carried out to confirm the findings of the preliminary test. The autoclaved liver diet was again used. The standard was fed at levels of 0·75, 1·5 and 3·0 I.U. daily and milks were given at presumptive levels of about 0·8 and 1·6 I.U. This was equivalent for spray-dried full-cream milk to 0·9 and 1·8 g. The corresponding levels for evaporated milk were 7·0 and 14·0 g. daily, but after a few days it became evident that the animals would not consume quantitatively the higher level and this was, in consequence, cut down to 10·5 g. Raw milk from the Institute's herd was also tested and the intake was adjusted daily by fluorimetric tests to supply the two levels of 0·8 and 1·6 I.U. The milk was fed to the nearest calculated 0·5 ml., the mean intakes being 6·6 and 13·2 ml. with variation for the lower level from 6 to 7·5 ml. The calculated intakes varied from 5·8 to 7·6 ml.

The results are given in Table 22. The agreement between biological and fluorimetric estimates, though still showing room for improvement in respect of evaporated milk, was now reasonably satisfactory for raw and for spray-dried milk.

C. STATISTICAL ANALYSIS

The method of computing the potencies of the milks in terms of the International Standard was, at the outset, that described by Irwin(40). It was found in the first experiment, however, that in five out of six cases the potency of any one type of milk was higher when fed at a high level than when fed at a low level; that is to say that doubling the dosage of a milk produced a greater improvement in growth than did doubling the dosage of International Standard. The attempt to calculate a single figure for the potency of each type of milk, irrespectively of the level at which it was fed, was therefore given up and separate figures for each level and for each type of milk were obtained by treating each level as a distinct specimen. This was most simply done by fitting a straight line to the mean responses to International Standard, regarded as a function of the *logarithms* of the dosage-rates, and adjusting the mean responses to the milk diets so that they agreed with this straight line. The method of least squares was used, each point being weighted according to the inverse square of the corresponding observed standard error of the mean. The *standard errors* of the *logarithms* of the potencies so found were calculated from Irwin's(40) formula and hence the *coefficients of variation* of the potencies themselves were deduced.

The apparent increase in potency on feeding at a higher level was observed in thirty cases, out of a possible thirty-two, in the whole series of experiments

Table 21, Exp. 6. *Mean gains in weight for standard and milk. 6 ♂♂ in each group. Vitamin B_1 potency of the milk estimated biologically and fluorimetrically*

		Gain in weight (g.)		Vitamin B_1 potency of milk			
				Biological estimation		Fluorimetric estimation	
		Gain	S.E.M.*	I.U./g. milk	C.V.† %	I.U./g. milk	S.E.M.*
Standard‡	0·75 I.U.	13·5	±3·58	—	—	—	—
	1·5 I.U.	43·3	±2·46	—	—	—	—
Evaporated milk I	6 g.	24·2	±6·77	0·160	18·0	0·142	±0·0035

* S.E.M. = standard error of mean. † C.V. = coefficient of variation. ‡ Second International Standard for vitamin B_1.

Table 22, Exp. 7. *Mean gains in weight for standard and milks. 6 ♂♂ and 6 ♀♀ in each group. Vitamin B_1 potency of the milks estimated biologically and fluorimetrically*

		Gain in weight (g.)				Vitamin B_1 potency of milks					
						Biological estimations				Fluorimetric estimations	
		Males		Females		Males		Females			
		Gain	S.E.M.*	Gain	S.E.M.*	I.U./g. milk	C.V.† %	I.U./g. milk	C.V.† %	I.U./g. milk	S.E.M.*
Standard‡	0·75 I.U.	6·2§	±2·04	6·8§	±2·40	—	—	—	—	—	—
	1·5 I.U.	35·0	±1·83	27·8	±1·11	—	—	—	—	—	—
	3·0 I.U.	51·2	±3·49	45·0	±4·34	—	—	—	—	—	—
Spray-dried milk IV	0·9 g.	3·3‖	±4·15	15·3	±3·06	0·73¶	16·2	1·06	15·6	0·95	±0·022
	1·8 g.	39·8	±3·54	43·2	±3·01	1·04	12·6	1·49	20·0		
Evaporated milk I	7·0 g.	28·3	±4·46	30·3	±3·59	0·19	13·6	0·24	15·3	0·13	±0·003
	10·5 g.	56·5	±2·18	44·7	±5·95	0·29	15·6	0·27	28·4		
						I.U./100 ml.		I.U./100 ml.		I.U./100 ml.	
Raw milk	6·6 ml.	−2·2‖	±2·52	13·2	±1·11	8·48¶	15·3	13·34	12·9	12·32	±0·253
	13·2 ml.	41·8	±5·15	43·3	±3·99	15·00	17·0	20·46	22·4		

Male-line equation: mean gain (g.) = 80·5 log dose + 18·0; female-line equation: mean gain (g.) = 62·1 log dose + 16·5.

* S.E.M. = standard error of mean. † C.V. = coefficient of variation. ‡ Second International Standard for vitamin B_1.
§ One animal lost weight. ‖ Two animals lost weight. ¶ By extrapolation.

(see Table 23, not including estimates from parabolas); in only one case did the reverse effect occur and in one case, not in the same experiment, no difference was found. Since many of these estimates of potency-ratios depend on extrapolation of the standardization curves, it would be better to assess the level of significance on a more conservative basis by considering only those cases which do not involve extrapolation. Of the figures, twelve in number, *not* marked with an obelisk in Table 23, only one does not exceed the corresponding dosage ratio. On the basis of number of cases, P therefore equals

Table 23. *Effect on apparent vitamin B_1 potency of milk produced by change in level of feeding*

				Males		Females	
Experiment	Type of milk and dosage		Dosage ratio (actual)	Biological dosage ratio	c.v.* %	Biological dosage ratio	c.v.* %
1	Roller-dried milk I	8 and 12 ml.	1·5	4·97†	41·6	1·70	15·1
	Spray-dried milk I	8 and 12 ml.	1·5	5·57†	46·3	1·93	20·4
	Evaporated milk II	8 and 12 ml.	1·5	1·50†	48·0	1·91†	11·9
2	Raw milk	4 and 8 ml.	2·0	5·11†	49·5	3·88†	60·0
(straight	Sterilized milk I	6 and 12 ml.	2·0	2·83†	70·4	11·17†	71·8
line)	Spray-dried milk II	6 and 12 ml.	2·0	6·50†	45·3	3·28†	38·9
	Evaporated milk III	8 and 16 ml.	2·0	5·51†	61·1	5·16†	79·0
2	Raw milk	4 and 8 ml.	2·0	5·00†	—	1·96†	—
(parabola)	Sterilized milk I	6 and 12 ml.	2·0	2·48†	—	2·97†	—
	Spray-dried milk II	6 and 12 ml.	2·0	11·22†	—	?	—
	Evaporated milk III	8 and 16 ml.	2·0	6·37†	—	?	—
3	Spray-dried skim milk	0·5 and 1·0 g.	2·0	5·41†	35·6	4·46†	48·7
(straight	Evaporated skim milk	4·5 and 9·0 g.	2·0	1·77	19·0	2·74†	29·6
line)	Spray-dried milk I	1·0 and 2·0 g.	2·0	2·18†	27·7	2·17†	33·4
3	Spray-dried skim milk	0·5 and 1·0 g.	2·0	2·19†	—	1·79†	—
(parabola)	Evaporated skim milk	4·5 and 9·0 g.	2·0	{1·74 {2·96	— —	— ?	— —
	Spray-dried milk I	1·0 and 2·0 g.	2·0	1·47†	—	1·40†	—
4	Evaporated milk II	5 and 10 g.	2·0	2·65	18·8	3·27	28·2
	Roller-dried milk I	1 and 2 g.	2·0	2·21	18·6	3·36	31·3
	Sterilized milk II	10 and 20 ml.	2·0	2·37	19·0	3·86†	39·2
7	Spray-dried milk IV	0·9 and 1·8 g.	2·0	2·84†	24·9	2·76	28·7
	Evaporated milk I	7·0 and 10·5 g.	1·5	2·24	19·0	1·69	28·5
	Raw milk	6·6 and 13·2 ml.	2·0	3·54†	25·8	3·06	30·1

* c.v. = coefficient of variation.

† Includes values obtained by extrapolation.

1:158. There is, therefore, no doubt as to the statistical significance of the phenomenon. It appears to occur about equally with all the types of milk used and with both sexes. It is slightly, but not significantly, less marked in the later experiments of the series.

Two explanations may be suggested. Either there is present in all these milks an accessory food factor not present in the basal diet, including possibly an inorganic trace element; or the addition of the major constituents of milk may improve the nutritive value of the diet as a whole. The quantities of milk involved were in some cases large enough to render the latter explanation by no means improbable. In order to diminish the effect the levels of milk

were kept as low as was consistent with good growth; but, in spite of this, the evaporated milk, being of low potency, had to be fed at such levels that the higher dosage sometimes amounted to nearly half the total intake of calories.

The difficulty appears to arise whenever the substance under test is of low potency and hence has to be administered in large quantities. It might possibly be avoided by adjusting the basal diet of the animals receiving the standard, in such a way as to make it more nearly similar to the mixture of milk and basal diet (cf. Copping & Roscoe (41)).

Both Irwin's method of calculation and ours, which is in principle equivalent to it, are founded on the logarithmic law of response. In seven out of

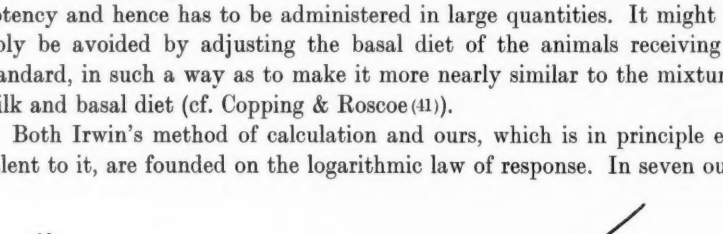

Fig. 5. Response of males in Exp. 3.

E.S. = Evaporated skim milk. S.F.C. = Spray-dried full-cream milk. S.S. = Spray-dried skim milk. The vertical strokes through the points referring to the International Standard represent twice the standard error of the mean response.

the ten cases in which it was possible to test the logarithmic law from our figures for response to International Standard the agreement was satisfactory; but in the remaining three cases (Exp. 2, females; Exp. 3, both sexes) a considerable curvature was found in the graph of mean response against logarithm of dosage. The curvature was in the same sense, concave downwards, in all three cases; that is, the responses both at the highest and lowest levels were considerably less than the amounts to be expected from a straight-line law (Fig. 5). Moreover, in the other seven cases, in which the straight-line law was confirmed within the limits of experimental error, the points would have been more closely fitted by a line having a slight, but not significant, curvature in the same sense (concave downwards) than by a straight line.

We accordingly decided to fit straight lines in every case but to duplicate the calculations by fitting curves in the two experiments where the deviation of the results from linearity was marked. The data were, of course, quite inadequate to decide what form of curve would fit best, as the experiments had not been designed for this purpose. The curve to be chosen has, however, to fit points only over a relatively short range, and as it has not to follow any changes of curvature, the choice is largely arbitrary.[1] Since the very considerable arithmetical labour is least when only a quadratic term, giving a parabola, has to be added to the equations, a parabola was fitted.

By fitting a parabola we do not wish to suggest that, if experiments were carried out under our conditions but with a considerably wider range of dosage levels, this form of curve would necessarily give the best fit over the whole range. Our experiments have merely shown that under certain conditions the linear relation of response to logarithm of dosage breaks down and must be replaced by a curvilinear relation of some type, the most convenient type for calculation being parabolic.

The occurrence of this curvature leads to one anomaly to which attention must be directed. In the three cases where the curvature is most marked, the highest point on the parabola actually falls within the range of the experiments; this means that if, as actually happened, the mean response to a level of milk exceeds the greatest value given by the parabola, it is not possible to assign any potency to that level; thus, for example, in the curve of response for females in Exp. 3, the mean increase in weight due to 2 I.U. of standard is 59·5 g.; by the time we reach the point corresponding to 4 I.U. of standard the curve has turned over and the response found is only 55·5 g. The highest point on the curve is calculated to be 61·4 g.; but the mean increase actually obtained with 9 ml. of evaporated skim milk is 68·8 g. and so cannot be fitted. Such cases are indicated (?) in the tables of potencies (Tables 18, 19). Where the mean response to a level of milk only just falls short of the crest of the standardization curve, two alternative estimates of the potency can be calculated but there is no means of deciding between the two and the verdict of the biological assay is very uncertain.

In Table 24 the results of all experiments of the series are summarized in so far as they refer to the comparison between biological and fluorimetric assays. The ratios between the potencies of each milk, as estimated by the two methods, are shown as percentages, together with their coefficients of variation and the probabilities that the ratio observed in each case would have arisen by sampling from a population in which the true ratio was unity. The coefficients of variation involve contributions arising from the uncertainties of both methods of estimation. The biological uncertainty was calculated by Irwin's method (cf. p. 175); the fluorimetric contribution was obtained from the standard error of the mean of all fluorimetric determinations made on samples of milk used in the feeding. The latter calculation was carried out

[1] The extreme dosage range (Exp. 2) is 16:1 corresponding to a range of log dose of only 1·204.

Table 24. *Statistical treatment of comparisons of biological and fluorimetric tests*

		Males			Females		
		Biological as % of fluorimetric value	c.v.* %	P†	Biological as % of fluorimetric value	c.v.* %	P†
		Exp. 1					
Roller-dried milk I	8 ml.	53‡	34·0	1 : 12	258	11·7	1 : 2000*b*
	12 ml.	177	30·6	1 : 12	286	8·5	1 : 14300*b*
Spray-dried milk I	8 ml.	35‡	41·8	1 : 33*a*	151	11·7	1 : 77*a*
	12 ml.	132	27·8	1 : 3	194	16·0	1 : 152*b*
Evaporated milk II	8 ml.	36‡	39·8	1 : 34*a*	115‡	8·1	1 : 8
	12 ml.	36‡	33·9	1 : 59*a*	146	9·7	1 : 100*b*
Raw milk	8 ml.	78‡	20·6	1 : 4	143	4·4	1 : 2000*b*
		Exp. 2 (straight line)					
Raw milk	4 ml.	150‡	40·2	1 : 3	120‡	40·4	1 : 2
	8 ml.	384	28·0	1 : 125*b*	233	49·5	1 : 10
Sterilized milk I	6 ml.	108‡	65·1	1 : 1	44‡	62·2	1 : 6
	12 ml.	150	33·4	1 : 4	245	36·8	1 : 22*a*
Spray-dried milk II	6 ml.	263	15·8	1 : 345*b*	590	38·6	1 : 178*b*
	12 ml.	840‡	47·4	1 : 185*b*	967‡	43·3	1 : 345*b*
Evaporated milk III	8 ml.	242	34·3	1 : 28*a*	375	54·0	1 : 30*a*
	16 ml.	646‡	57·8	1 · 73*a*	1040‡	57·1	1 : 147*b*
		Exp. 2 (parabola)					
Raw milk	4 ml.	153‡	—	—	157‡	—	—
	8 ml.	341	—	—	155	—	—
Sterilized milk I	6 ml.	117‡	—	—	108‡	—	—
	12 ml.	145	—	—	158	—	—
Spray-dried milk II	6 ml.	249	—	—	364	—	—
	12 ml.	1408‡	—	—	?	—	—
Evaporated milk III	8 ml.	248	—	—	304	—	—
	16 ml.	783‡	—	—	?	—	—
		Exp. 3 (straight line)					
Spray-dried skim milk	0·5 g.	39‡	33·3	1 : 22*a*	42‡	47·3	1 : 14
	1·0 g.	104	18·9	1 : 1	95	21·9	1 : 15
Evaporated skim milk	4·5 g.	145	13·6	1 : 23*a*	132	17·5	1 : 5
	9·0 g.	128	16·1	1 : 6	191	23·1	1 : 15
Spray-dried milk I	1·0 g.	70‡	34·8	1 : 3	76‡	25·5	1 : 3
	2·0 g.	76	16·9	1 : 7	83	27·4	1 : 2
		Exp. 3 (parabola)					
Spray-dried skim milk	0·5 g	91‡	—	—	93‡	—	—
	1·0 g.	100	—	—	83	—	—
Evaporated skim milk	4·5 g.	145	—	—	124	—	—
	9·0 g.	127	—	—	—	—	—
		215	—	—	?	—	—
Spray-dried milk I	1·0 g.	108‡	—	—	105‡	—	—
	2·0 g.	79	—	—	75	—	—
		Exp. 4					
Evaporated milk II	5·0 g.	147	11·1	1 : 56*a*	127	15·7	1 : 5
	10·0 g.	193	19·0	1 : 63*a*	206	19·5	1 : 100*b*
Roller-dried milk I	1·0 g.	141	11·9	1 : 33*a*	123	15·5	1 : 5
	2·0 g.	156	12·9	1 : 66*a*	205	22·3	1 : 62*a*
Sterilized milk II	10 ml.	115	13·7	1 : 3	78‡	30·3	1 : 2
	20 ml.	136	10·6	1 : 37*a*	151	16·9	1 : 263*b*
		Exp. 6					
Evaporated milk I	6 g.	113	18·0	1 : 2	—	—	—
		Exp. 7					
Spray-dried milk IV	0·9 g.	77‡	16·2	1 : 7	112	15·6	1 : 2
	1·8 g.	110	12·6	1 : 2	157	20·0	1 : 18
Evaporated milk I	7·0 g.	146	13·6	1 : 33*a*	185	17·3	1 : 143*b*
	10·5 g.	223	15·6	1 : 386*b*	208	28·4	1 : 31*a*
Raw milk	6·6 ml.	69‡	15·3	1 : 21*a*	108	12·9	1 : 4
	13·2 ml.	122	17·0	1 : 4	166	22·4	1 : 18

* c.v. = coefficient of variation.

† P = probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population. *a* = over 1 : 20, *b* = over 1 : 100.

‡ = biological value extrapolated.

before we realized that in one or two cases duplicates were involved, and hence leads to a slight underestimate of the actual fluorimetric error. As, however, the term in question turned out to be extremely small by comparison with the term arising from biological variability, and as the two terms enter the formula as squares, the fluorimetric term was omitted altogether.

Since the formulae for computing coefficients of variation of potencies derived by fitting parabolas are very cumbersome and since none of our deductions depend essentially on the exact values of these coefficients, we did not calculate them in all cases and we have not included them in the tables. The order of accuracy involved is for moderate dosages the same as that given by the straight line; for the higher dosages, the parabola is less accurate than the straight line and may give very unreliable results or no result at all (cf. p. 179); for the lower dosages, the parabola usually gives somewhat smaller sampling errors than the straight line.

On surveying Table 24, the most noticeable point is the improvement of agreement between the biological and fluorimetric estimates when the latter are adapted to measure total (Exps. 3–7) rather than "free" vitamin B_1 (Exps. 1, 2). The subsequent modifications of technique have produced small but appreciable improvements in the agreement and in the last experiment one may say that, apart from the still outstanding effect of difference in level of feeding, the two methods are reasonably concordant for raw and dried milks. Evaporated milk gives less satisfactory results than dried milk in the last as in previous experiments.

SUMMARY

Part I

1. A method for the estimation of vitamin B_1 in milk by a modified Jansen test involving takaphosphatase treatment is described in detail.

Part II

2. It is shown by ultra-filtration experiments, by treatment with pepsin and takaphosphatase, with trichloroacetic acid and with fuller's earth that vitamin B_1 is present in milk unesterified and phosphorylated and that both forms may be freely diffusible or combined with protein either loosely or by firmer bonds.

Part III

3. The effect of the stage of lactation on the amounts and partition of vitamin B_1 in milk has been studied. Samples of colostrum and early milk were found to contain large amounts of cocarboxylase (up to 80 % of the total). Hardly any cocarboxylase was present in mid or late lactation milks. The total vitamin B_1 content was 60–100 μg./100 ml. for colostrum, up to 60 μg./100 ml. for early milk and 30–40 μg./100 ml. for mid and late lactation.

4. A marked negative correlation exists between the cocarboxylase content of cow's and goat's milk and their alkaline phosphomonoesterase

titre. As this enzyme cannot dephosphorylate cocarboxylase it is probably accompanied in milk by other enzymes more suited to this process.

5. The suggestion is put forward that the vitamin B_1 of milk is mostly derived not directly from the blood but from the secretory cells of the mammary gland.

6. Feeding and season have little or no effect on the vitamin B_1 content of milk.

7. Fluorimetric measurements of riboflavin have shown that its content is some 3–4 times higher in colostrum than in later milk.

8. A study from March to June 1938 of the riboflavin content of the milk of a group of Shorthorn cows and a comparison of the milk of a group of cows kept indoors on a winter ration with that of another group on pasture, carried out from March to December 1939, have shown that the riboflavin content of milk is increased by summer feeding. The mean values were about 110 μg./100 ml. in winter and 150 μg./100 ml. in summer.

Part IV

9. Seven separate experiments were carried out in which the fluorimetric method of assay of vitamin B_1 was compared with the biological test. Raw, sterilized, spray- and roller-dried and evaporated milks were tested and the results were subjected to statistical analysis.

10. In the first two experiments only the "free" vitamin B_1 was measured in the fluorimetric tests and the biological assays yielded in consequence definitely higher values.

11. When the fluorimetric test measured the total vitamin B_1, and the vitamin B_2 complex in the biological assay was supplied as autoclaved liver the agreement, especially at lower levels of milk feeding, was satisfactory for all but the evaporated milk.

12. The apparent potency of all milks as measured biologically was systematically higher when milk was fed at a high level than when it was fed at a low level. Evaporated milk, owing to its relatively low potency, had to be fed in larger quantities on the dry matter basis than other milks. It is possible that the discrepancy between fluorimetric and biological assays at high levels of milk feeding may have been due to a deficiency of some essential factor in the basal diet but the beneficial effect of the major constituents of milk cannot be ruled out.

13. In all biological assays the International Standard for vitamin B_1 was used and logarithmic response curves were fitted. Though a straight line fitted most of the results satisfactorily, it was found that a better fit could be obtained in all cases by means of a parabola concave downwards.

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637·333·1

259. A NOTE ON A SIMPLE AND ACCURATE VISCOSIMETRIC FORM OF RENNET TEST

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(With 3 Figures)

Since publication(1) the apparatus previously described by the authors has been altered and improved. The alterations have been concerned mainly with excluding draughts and securing a better form of support. In using the apparatus we have found that the absence of vibration is important for the agreement of duplicates. It would seem that vibration tends to break the cohesion between the curd and the surface of cylinder *A*. For this reason a part of the laboratory as free as possible from vibrational disturbances was chosen and the support fixed to the wall.

The procedure already described was adhered to, except that the rennet was added to the milk in the beaker and stirred in before lowering cylinder *A* into the milk. As before, adding the rennet and starting the pendulum to oscillate occupy about 15 sec., and the time of coagulation is taken as the period between the addition of one-half of the rennet and the moment the pendulum comes to rest.

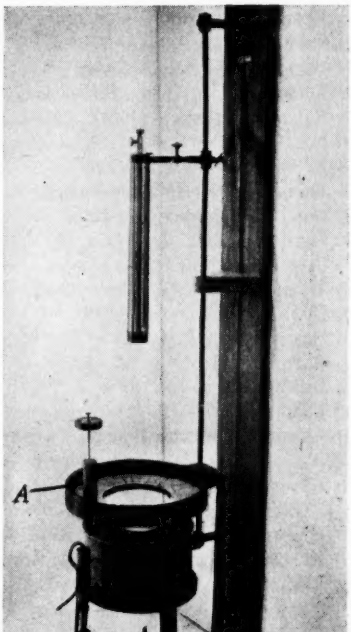

Fig. 1.

Fig. 1 shows the position of the parts of the apparatus immediately before the rennet is added. After the rennet has been added and stirred in, the cylinder is lowered into the milk and started to oscillate as before. The glass plate is then placed over the scale and pointer as shown in Fig. 2. For convenience this plate is in two pieces. Finally, the brass tube is fully extended and during the remainder of the test, that is, till the pointer comes to rest, the parts are assembled as shown in Fig. 3. It should be noted that the surface of *A* must be perfectly clean, and it is now recommended that the cylinder be

placed in petroleum ether between each test. Table 1 gives a comparison between original apparatus and the improved form in respect of coagulation times, means and standard errors. These results were obtained with the use of the same milk sample throughout and the diluted rennet solution was freshly prepared after every fifth test in each series.

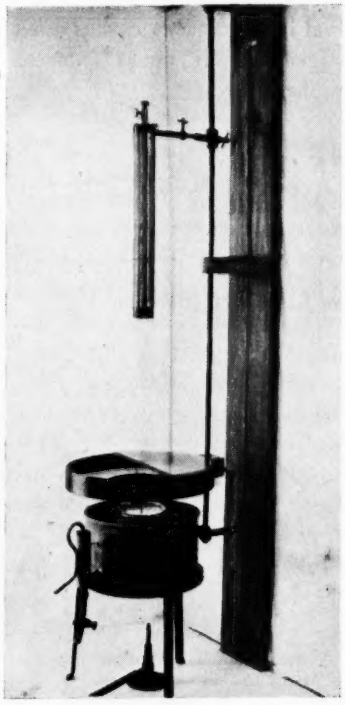

Fig. 2.

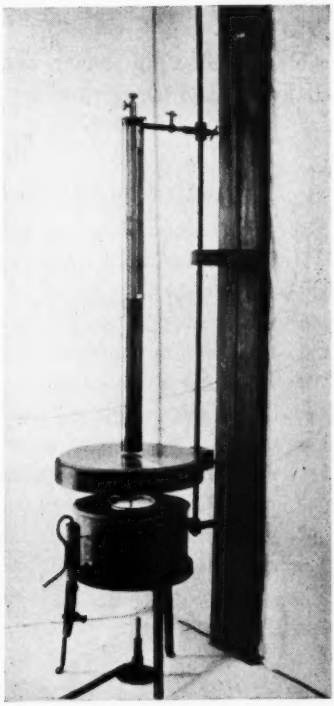

Fig. 3.

Table 1. *Coagulation times*

Original apparatus				Improved apparatus			
min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.
2 45	2 44	2 47	2 45	2 44	2 44	2 45	2 44
2 44	2 47	2 49	2 45	2 50	2 47	2 48	2 48
2 48	2 47	2 47	2 50	2 44	2 43	2 43	2 50
2 45	2 49	2 48	— —	2 43	2 49	2 44	2 47
2 49	2 52	2 52	— —	2 43	2 45	2 45	— —
	Means		2 47·39				2 45·58
	Standard errors		±2·41				±2·37

While the differences in use between the two forms of apparatus cannot be considered statistically significant and while good duplicates are possible with the original apparatus, the protected form is superior in evenness

of oscillatory movement and definiteness of end-point and therefore tends to give more satisfactory results.

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637·333

260. AN OBJECTIVE MEASURE OF THE CONSISTENCY OF CHEESE CURD AT THE PITCHING POINT

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(With 6 Figures)

Introduction

The importance of finding some objective and quantitative measure of the consistency of cheese curd at various stages in manufacture, in view of the limitations of even the most experienced cheese-makers to reproduce their judgements accurately, has already been pointed out by Coppen (1), while Scott Blair (2) has described an apparatus for measuring the firmness of curd at the cutting point. At this early stage, the curd has comparatively simple rheological properties, at least when exposed to small stresses for short periods. It behaves very nearly as an elastic solid, and the shear modulus, which can be easily determined, gives a single criterion of its firmness, which, although not the only factor judged by the cheese-maker, is in itself of sufficient importance to serve as a measure of the self-consistency of his subjective opinions.

It is realized, however, that in practice, quite large differences can be tolerated in the firmness of curd at cutting without serious effects on the quality of the cheese, whereas a much more exact definition of the condition suitable for pitching is required. Especially under war-time conditions, an objective criterion of this consistency is most desirable.

No deep psychological investigation is required to establish that the cheese-maker is influenced, or believes himself to be influenced in judging this consistency, by a number of properties, and firmness, spring, resilience and homogeneity of moisture distribution within the curd-lump are among the many complex contributory factors. Yet for practical purposes, since all these properties cannot be rapidly measured during the scalding process, some simple criterion is required which may be taken as an overall measure of firmness. In a case of this kind, when a judgement depends on a composite mixture of physical properties, the only possible criterion of the effectiveness of any physical test is its correlation with the subjective judgement itself. This judgement is subject to variations which are themselves the object of investigation. The only way, therefore, in which the value of a test of this sort can be fully assessed, is to collect a very large number of data and correlate them with the most frequent judgements of the experts. In the meantime, if any physical property can be measured which seems to be so correlated, there is every reason why such a method should be made available as early as

possible so that the maximum number of data may be collected. In looking for such a property, theoretical considerations must not be forgotten, and the opinion of the expert, although to be regarded with some caution, should be taken into account.

The simplest of such properties would be moisture content, but for two reasons this is unsuitable. First, the changes take place very rapidly, and no test for moisture is available which can be carried out within a minute or so, and secondly, as will be shown later, however carefully samples are taken, moisture contents determined afterwards on samples taken from the vat during scalding are not closely reproducible, doubtless because of difficulty in ensuring reproducible conditions for the draining of free whey from the curd.

The second most suitable property to attempt to measure appeared to be the compressibility determined by loading the material, either restricted in a gauze cylinder or as a free cylinder of curd. Here the difficulty lies in the fact that the condition of packing of the pieces of curd is important and is not easily controlled, and further that in the case of a "free" cylinder, the most significant part of the compression takes place under the weight of the cylinder itself before any measurements can be made.

Experiments on compressing individual curd lumps in a micro-apparatus gave excellent results for each individual lump, but the heterogeneity of the material is such that a great many such experiments would have to be done before a reliable mean figure could be obtained.

As a result of experiments carried out during a period of two years, a partly empirical solution to the problem has been found. A small cylindrical sieve of suitable mesh (16 per in.) can be filled with curd by drawing it through the stirred contents of the vat. The curd is then gently caused to slide out of the inverted sieve to form a consistent cylinder of packed curd and this slumps into a shapeless mass only when the sample is taken during the earliest stages of the scalding process. Such cylinders are smooth and regular wherever they have been in contact with the sieve. The surface at the open end of the sieve can be smoothed by sliding in a gauze disk and inverting the cylinder (see Fig. 1), then reinverting, and finally inverting again on to a flat surface and allowing the curd to slide out as before.

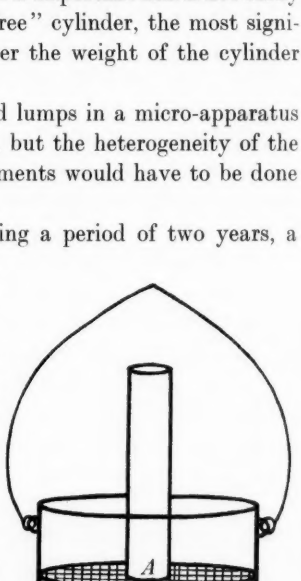

Fig. 1.

If these inversions are performed at regular times after the cylinder is first filled in the vat (5, 20 and 35 sec. respectively are convenient times) closely reproducible cylinders can be obtained, except only that in our experiments the total amount of curd removed from the vat varied somewhat

whatever precautions were taken. In doing compression tests, it was observed that during the scalding process, the heights of such cylinders (h) measured at a definite time (50 sec.) after the final inversion, progressively increased as the curd toughened until near the pitching point when there is very little change in height after the cylinder has left the gauze mould. This height was not in itself an adequate criterion of the progress of scalding, since the total amount of curd is not closely reproducible. If the gauze cylinder with the curd in it is weighed on a spring balance[1] 7 sec. after the second inversion (the exact time is important, since some free whey is still draining from the cylinder) it is found that there is a tendency for this weight (W) to fall during scalding, though again not very regularly.

It was found, however, that if the effects of chance differences in the amount of curd collected are eliminated by dividing W by h, the ratio W/h not only gives a very regular curve when plotted against time in minutes before pitching (t), but that the same cheese-maker tends to give judgements on the best consistency for pitching a given type and size of cheese (neglecting adjustments to allow for abnormal acidity) which correspond with a very small range of W/h values. The consistency of these figures is more marked in some varieties than in others. If W/h can be shown to be related to the sort of properties which the cheese-maker aims at assessing, and if it is not unduly affected by such variation in manufacture as reasonable differences in size of cut and methods of scalding, then in view of its reproducibility and simplicity (the whole test takes less than 1 min. to complete) we may regard W/h as a potentially useful criterion for pitching.

Experimental procedure

Stirring the vat vigorously, the experimenter draws the gauze sieve through the curd in a horizontal direction, finally turning the hand to bring it to the surface open end uppermost. A stop watch is started when the sieve leaves the vat. The curd is inverted on to the disc after 5 sec. and reinverted at 20 sec. The sieve is weighed on the balance at 27 sec. It is inverted again on to a small metal platform (a moisture tester tin is effective) which stands in a flat petri dish to catch the draining whey. A pointed meccano rod working on a worm (part of the apparatus described in the earlier paper (2) was actually used) is screwed down towards the top of the curd cylinder on which rests a light xylonite disk.[2] The point at which the rod just touches the disk is most easily seen if a very small polished metal disk (about 1·3 cm. diameter) is stuck to the centre and on top of the xylonite disk. The height of the cylinder is afterwards read off directly on a scale by means of an indicator attached to the meccano rod, the reading being taken at 50 sec. An electrical device has been

[1] A 100 g. spring-balance is used. The gauze cylinder weighs 20 g. and by means of an improved pointer and a "counterpoised" scale read through a magnifying glass, a sufficiently accurate weighing can be quickly made (to the nearest 0·5 g.).

[2] We are indebted to Messrs Halex, Ltd. and especially to Dr W. G. Wearmouth for supplying this disk as well as for other similar materials used in our experiments.

constructed to ring a bell at the required intervals so that one person can perform the whole test. If it is desired to correlate the W/h value with moisture, a moisture sample is taken at 60 sec. by means of a cork-borer of diameter 1·4 cm. which is pressed into the centre of the cylinder, the sample being caused to slide into a small weighing-bottle. The moisture contents are afterwards determined[1] in the Carter-Simon Rapid Moisture Tester (3) at a temperature of 155° C. for a time of 9 min., which for this material, corresponds to about $1\frac{3}{4}$ hr. in a steam oven at 98° C. This method is, like all moisture tests on such materials, arbitrary, but is as satisfactory as any other and has the advantage of being very rapid. There is nothing of special value in the method described for measuring h and in other laboratories or dairies, where different equipment happens to be available, doubtless other methods could be used equally effectively.

Significance of W/h

During the scalding process, a number of colloidal changes are taking place in the curd, and these produce in their turn many rheological changes. Soon after cutting, the curd lumps are soft and gelatinous. When formed into a cylinder, these lumps readily deform so as to fill any gaps in the structure except those small enough to be occupied by water held by capillary forces. There are few or no air holes in the material. As scalding proceeds, there is a syneresis of whey causing a shrinkage and hardening (increase in elastic moduli and resilience) of the curd, so that the cylinder tends more and more to keep its shape when left to stand, the particles fitting into some type of packing such that free whey drains off and air spaces are formed between the granules. At the condition when pitching is advisable (in the case of Cheddar and several other varieties) the cylinder keeps its shape comparatively well, and quite a high proportion of its volume is occupied by air. The superficial density is, therefore, much lower than at the start, and W/h combines a measure of this density with that of the tendency of the cylinder to "slump down" under its own weight. In the earlier stages of scalding, the latter effect preponderates, since differences in density are small and W is therefore approximately proportional to the height of the curd while still restricted in the sieve. Thus W/h measures chiefly the strain[2] produced by gravity on removing the restraint of the sieve. We have attempted to measure the height of the curd within the sieve, but this we could not do with sufficient accuracy.

Although the whole process of scalding can be followed by observing the changes in W/h, it must be realized that the significance of W/h is changing, so that, near the pitching point where h is nearly proportional to volume, we are measuring an overall density, giving the extent to which air has replaced the whey which is free to escape from the cylinder. At this stage, less water is

[1] We wish to thank Mr A. Wagstaff for making these determinations.

[2] The correct expression for this strain is $\log_e (h_0/h)$, where h_0 is the "restricted" height, and $h_0 = W/\pi R^2 \delta$, where R is the radius (2·4 cm.) and δ the density of the material.

bound mechanically and chemically by the curd, although the water which is so bound may be held even more strongly than before.

It thus appears that W/h is in part empirical, but has some claim theoretically to represent a criterion for pitching. Although moisture tests are not as reliable as are the determinations of W/h, it is of interest to enquire how far W/h and moisture are correlated.

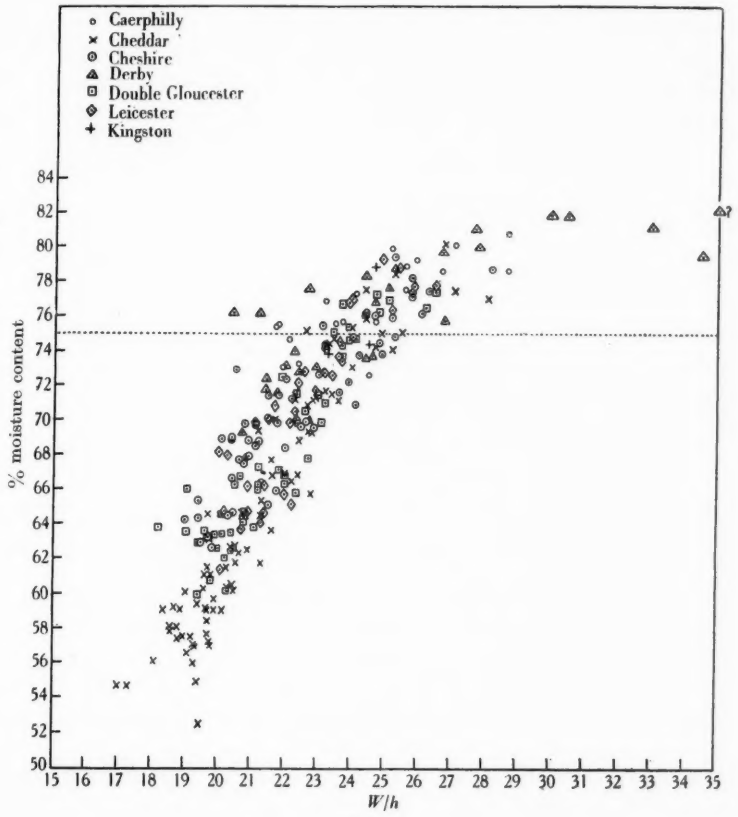

Fig. 2.

It is clear that the relationship between consistency and total moisture content must be dependent on an approximately standard method of heating. Thus a process in which heat is applied very drastically at the start might well form a firm surface layer round the curd particles, giving them a superficial rigidity not in keeping with their total moisture content. Determinations of moisture contents above 75 % were considered somewhat unreliable, and at moistures below this, on 197 determinations for cheese receiving normal heat treatment, the correlation coefficient between W/h and moisture content was 0·87, a remarkably high value. The scatter curve is shown in Fig. 2. It will be

observed that the curve flattens out considerably at high moisture contents. The correlation coefficient takes no account of the differences in cheese type: thus it is clear that, at any rate at lower moisture contents, the data for Cheddar fall lower on the diagram than those of other types. In fact the different treatments for the different types of cheese are bound to influence to some extent the W/h moisture relation.

The method of taking moisture samples is admittedly unsatisfactory and could perhaps be improved. If this were done, the correlation coefficient would doubtless be still higher. In Fig. 3, the full line gives the calculated best straight line representing the data in Fig. 2, and the points plotted are those from a special experiment in which heat treatment was abnormal. In this

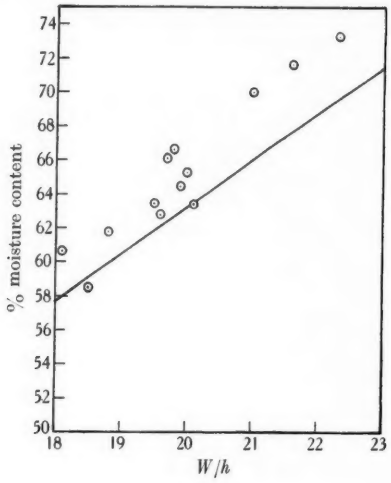

Fig. 3.

experiment the temperature was raised rapidly to 96° F. and held approximately constant for 25 min. The temperature was then raised to 104° F. after which it was again kept approximately constant (103–4° F.) until pitching. The discrepancy between these data and the normal curve, although comparatively small, is significant.

The effect of size of cut on W/h values is of interest. An experiment was carried out in which three small cheese (Cheddar type) were made in buckets simultaneously in the same vat. A was cut as coarse as was consistent with the carrying out of the test, and C as fine as is reasonable for Cheddar, B being cut to an intermediate size, but approximating more nearly to A than to C. In Fig. 4 are given the W/h-moisture curves, and in Fig. 5 the W/h-t curves.

It will be observed that the W/h-moisture relation is unaffected by the size of cut, and that the progressive fall of both these properties with time, although more rapid the coarser the cut, approaches at the pitching point a unique

value independent of size of cut. We therefore conclude that W/h is a remarkably stable criterion of consistency at pitching, even allowing for fairly wide divergence in the manufacturing process.

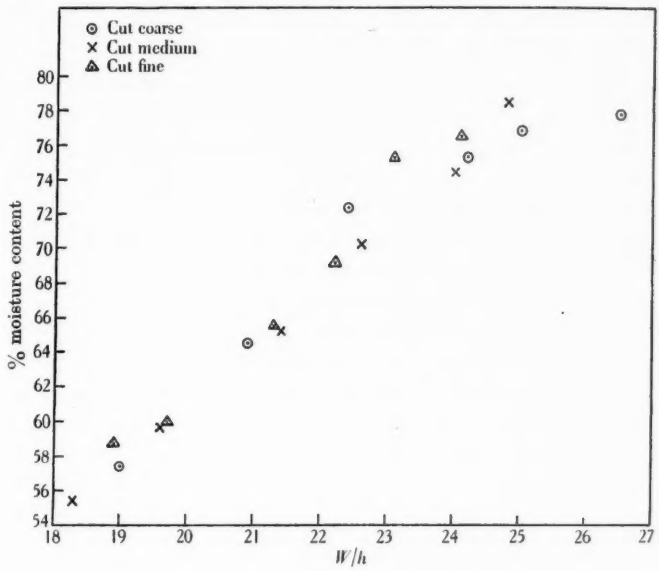

Fig. 4.

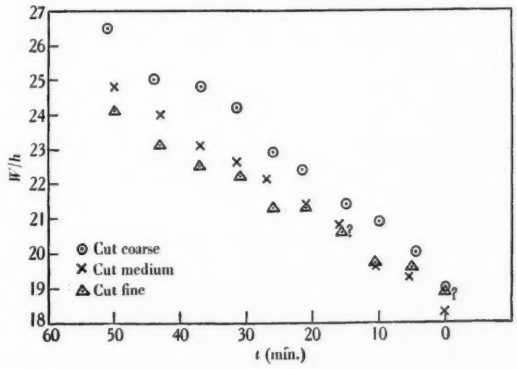

Fig. 5.

Effect of acidity, fat content of milk, etc.

It is clear that the shape of the W/h-t curve will be greatly influenced by differences in acidity. We have already obtained considerable confirmation of this, but quantitative data are not easy to get on account of the inaccuracy of the titration method combined with the comparatively small change in

titratable acidity which takes place between cutting and pitching. The whole of this change is only about five times the normal error of the titration. It is hoped that it will be possible to improve this situation by developing a simple and rapid method for determining the *p*H of whey.

The effect of varying fat contents and other chemical conditions are clearly important, and will form the subject of further investigations.

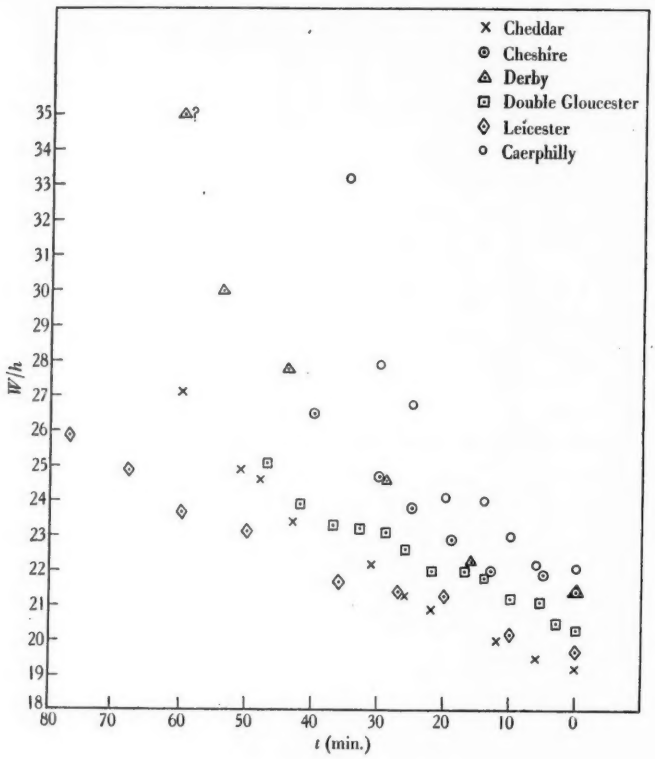

Fig. 6.

Types of cheese to which the W/h test is applicable

It is intended to make an intensive study of a very large number of cheese by this method. We have to date made satisfactory tests on thirty-two Cheddar, as well as on twenty-three Cheshire, six Leicester, fourteen Double Gloucester, one Kingston, six Caerphilly and eleven Derby cheese (see Fig. 2). The method is, without modification, applicable to all these types, though a larger sieve might perhaps be advantageous with the coarser cut varieties. It is likely that the absolute values of W/h are not altogether independent of the size of the sieve. There is every reason to suppose that, with or without minor modifications, a number of other varieties will be satisfactorily tested.

In the factory in which the tests were carried out under the directions of a single skilled cheese-maker, the mode of the distribution curve for W/h at pitching point for Cheddar occurs at about 19·0 on the data obtained so far. A typical W/h-t curve for a Cheddar pitched at 19·0 is given in Fig. 6, and curves for Leicester, Double Gloucester, Derby, Caerphilly, and Cheshire varieties are also included, though we are not yet in a position to say for certain that they are really typical of these varieties. The Cheshires particularly show large individual differences due to differences in the size of cheese. The curves serve to show, however, the kind of differences to be expected.

Summary

1. A method is described for measuring the consistency of cheese curd during the scalding process which has been found satisfactory with Cheddar, Cheshire, Leicester, Double Gloucester, Caerphilly, Kingston and Derby cheese and is probably applicable to some other varieties.

2. The property (W/h), taken as a criterion of consistency, can be measured within less than 1 min. and is highly correlated with moisture content except when abnormal scalding conditions produce an uneven distribution of moisture in the curd. The size of cut does not affect this correlation within wide limits, though both moisture content and W/h are regularly affected by size of cut until the curd is approximately ready to pitch, at which point both properties show unique values independent of size of cut.

3. When the curd is nearing the pitching point, W/h measures the extent to which a standard cylinder of curd loses whey, the liquid being replaced in the solid structure by air. At an earlier stage in the scalding process the capacity of the curd to retain its shape under the force of gravity also affects W/h.

4. Although the cheese-maker judges the consistency of curd suitable for pitching by a complex mixture of properties, it is believed that W/h will serve as an effective single-value criterion of consistency, suitable for judging pitching time if the acidity is also known.

The authors wish to express their best thanks to Miss D. V. Dearden for carrying out the special experimental cheese-making required for this work, and also to Prof. E. Capstick and the Staff of the British Dairy Institute, and especially Miss V. E. Cheke, for helpful co-operation in collecting data and giving expert advice.

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION D. THE NUTRITIONAL VALUE OF MILK AND MILK PRODUCTS

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III. Nutritional value as estimated in laboratory and field experiments.
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      (i) General.
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I. INTRODUCTION

THE review covers the two years from the beginning of 1938 to the beginning of 1940. The number of papers dealing with various aspects of the food value of milk increases steadily from year to year. The scope of the review has again been restricted to cow's milk, and no attempt has been made to discuss either the various modified milks used

in infant feeding or the role of milk and milk products in the feeding of poultry, while the value of milk for calves is covered not by this section but by section A of the biennial reviews. Nevertheless, a number of valuable papers could not be included. The papers which have not been read in the original are marked with an asterisk in the References.

II. GENERAL

No comprehensive articles devoted to the nutritive value of milk have come to the reviewer's notice. Much interesting information will be found in the excellent book on *The Englishman's Food*, by Drummond & Wilbraham (*a*). Sommer's book (*b*) contains a chapter on the nutritive value of milk. A good popular article has been written by Irwin (*c*). References to reports and summaries dealing with specific aspects of the subject under review are given in the relevant sections of the present paper.

III. NUTRITIONAL VALUE AS ESTIMATED IN LABORATORY AND FIELD EXPERIMENTS

A. Raw milk (and butter in the cases of vitamins A and D)

(*a*) *General*

The study of fluctuations in the nutritive value of milk by the technique of exclusive milk feeding has been continued in the United States and in this country. Earlier work on this subject was reported in the two previous reviews (1, 2). According to the American workers the changes are connected with the concentration in milk of the "grass-juice factor" distinct from all known vitamins. To study this factor in grasses and silages and in milk they use now, instead of rats, guinea-pigs, which are said to be more sensitive (3, 4, 5, 6, 7). According to the earlier reports (3, 6) mineralized milk alone, even from cows on pasture or receiving A.I.V. or molasses alfalfa silage, was insufficient to keep guinea-pigs alive, but in the latest publication (7) good growth was reported of one guinea-pig receiving summer pasture milk and of two receiving milk produced by cows on phosphoric acid alfalfa silage. This was in contrast to the poor performance of guinea-pigs given milk produced on molasses clover-timothy silage and molasses alfalfa silage. Milks produced on a winter ration and on A.I.V. and molasses alfalfa silage were also fed by Hegsted *et al.* (6) to rats but with inconclusive results. In this country Bartlett *et al.* using specially prepared (8) rats fed exclusively on mineralized milk, failed to find any difference between milks produced by cows receiving as dietary supplements mangels, artificially dried grass, sprouted maize or kale (9), or between milk produced simultaneously on a winter ration and on good early summer pasture (10). In their experience guinea-pigs survived no longer on the summer than on the winter milk. These results are in agreement with the earlier work from the same laboratory reviewed in (1) and are further supported by experiments on the processed milks reported on p. 208. They show that under conditions obtaining in England the method of exclusive milk feeding cannot be used as a means of detecting nutritive difference between milks.

Richardson & Hogan (11) maintained four generations of female rats on mixtures of raw milk and whole-milk powder in the proportion of 1 : 1 and later 2 : 1, supplemented with iron, copper, manganese and iodine. The females were mated with stock colony males. Their reproductive performance was inferior to that of stock colony controls. Mueller (12), in confirmation of earlier work (1), found that rats receiving mineralized milk containing 4 % of cocoa grew less well than rats receiving mineralized milk alone, but that addition of cod-liver oil and brewer's yeast to both diets cancelled the difference.

Sasaki & Kandatsu (13) report that raw milk equivalent to 1·5–2 g. of solids daily has the same effect as dried yeast or yeast extracts in preventing injury in rats fed excessive amounts of cane sugar. Heating destroys this property in milk.

Campbell (14), who is studying the effect of diet on the resistance of rats to oxygen want, as at high altitudes, found that milk is not a suitable food for such conditions, probably owing to its high protein content.

(*b*) *Vitamins*

(i) *General.* Useful information on the vitamin content of milk and milk products will be found in Fixsen & Roscoe's (15) tables, and an up-to-date review of the effect of hay, artificially dried crops and silage on the vitamin content of milk is given in Watson's book (16). The book of Gstirner (16*a*) gives a useful compilation of methods of chemical assay of vitamins, many of them applicable to milk. Joachimoglu & Sotiriadou (17) have studied the vitamin A, B_1, C and D content of milk in Athens.

(ii) *Vitamin A and carotene.* Judging by the number of published papers vitamin A and carotene have attracted more attention than the other vitamins of milk. Chemical and physical methods of estimation of vitamin A and of carotene in milk and milk products have aroused much interest. Olson *et al.* (18), Willstaedt & With (19) and van Bever (20) have described methods of direct estimation of both factors in milk. The former two first extract the fat by a modified Röse-Gottlieb technique. This is then saponified and measurements are either carried out spectrophotometrically (18) or by photometer measurements, using the antimony trichloride reaction for vitamin A (19). Van Bever (20), who measures only vitamin A, saponifies milk directly and estimates as in (19). His is a very careful study of the application of the Carr-Price reaction to milk and butter. Wolff's paper (21) should also be consulted. A rapid photoelectric method of estimation of carotene in milk fat has been described by Hand & Sharp (22). Chevallier & Manuel (23) use destructive irradiation with ultraviolet light for the determination of vitamin A in milk. They report surprisingly that only one-quarter of the vitamin A of milk is recovered in the butter while three-quarters are left in the separated milk. This is contrary to the generally accepted view (cf. e.g. (19)). Demarest (24) is of the opinion that this method cannot be at present applied to the non-saponifiable residue of milk. Small-scale chromatographic analysis suitable for the study of milk carotenoids is described by Willstaedt & With (25).

Though normally only the β-isomeride of carotene is present in milk fat, this is accompanied by the α variety if cows have access to carrots (26, 27), and in general the carotenoids of butter will vary with those ingested by the cow. Thus Quackenbush *et al.* (28) have shown that epiphasic yellow pigments devoid of vitamin A activity, formed in lucerne silage by the action of acid, can be recovered from the butter of cows receiving the silage. Unless it is therefore known that β-carotene is largely predominant in the fodder, a chromatographic analysis of the butter is advisable (26, 27).

It has been shown now by Zechmeister & Tuzson (29) that, contrary to earlier views (30), the adsorption process in chromatographic analysis does not cause isomerization of α- and β-carotene, and this has been confirmed by Carter & Gillam (31). Difficulties in the spectrophotometric assay of vitamin A and carotene in butterfat are pointed out by Meigs (27), and peculiarities of the extinction coefficient of butter vitamin A in various solvents have been discussed by Gillam & El Ridi (32).

A good review of industrial methods of manufacture of carotene and of its possible utilization as a colouring matter for butter will be found in (33).

More papers have appeared dealing with the problem of the relative biological efficiency of vitamin A and carotene. Fraps & Kemmerer (34), on the basis of extensive

spectrophotometric tests, have come to the conclusion that 1 μg. of butter vitamin A is equal to 8 international units as against 1·68 I.U. for the same quantity of carotene. It must be said that the biological estimations on which these calculations were based were expressed in Sherman-Munsell units apparently without direct comparison with the international standard for vitamin A. Kraybill *et al.* (35) found that β-carotene was less well utilized when dissolved in butterfat than in maize oil; the finding that the lowest utilization was from cotton-seed oil seems to reverse their earlier views (36). On the other hand, Lease *et al.* (37) obtained equal growth responses when rats were given 1 μg. daily of carotene dissolved in cotton-seed oil, soya-bean oil, lard, decolorized butterfat, coco-nut oil or crude earth-nut oil. Whatever the specific effect of butterfat, the evidence is accumulating to show that vitamin A is in general more efficiently utilized than carotene (38,39,40), that the relative efficiency of utilization varies with the level of intake (38,39), that the response of a depleted animal to small doses of vitamin A or carotene is no indication of the response of a normal animal to larger doses, and that the adoption of carotene as the international standard for vitamin A—though perhaps unavoidable—has very serious drawbacks.

Hauge *et al.* (41,42) have continued their studies of the vitamin A depressing factor in soya beans. It was found that though it lowers the vitamin A content of butter the factor does not affect carotene. It apparently acts by interfering with the conversion of carotene to vitamin A and by inhibiting the transfer from food to milk of vitamin A ingested as such. A study of the active principle itself is in progress.

Studies of the seasonal variations of vitamin A and carotene or of vitamin A activity of milk and butter have been reported from Belgium (43), the United States (44,45,46), all four on market milk supplies, for milk from Finnish farms (47) and for Danish and Scotch butters (48) as well as for butter in India (49,50). Butter produced under drought conditions and butter from irrigated areas in Idaho were also studied (51,52), while figures for milks bought in London are quoted in Coward's book (53). The investigations detailed in (44), (48), (52) and (53) were carried out by biological methods; the remainder by chemical and physical tests. As usual, marked differences are noticeable, the American milk (44) ranging from 22 I.U./g. fat in April to 80 I.U./g. in August, Scotch butter (48) from 8 I.U./g. in April to 29·9 I.U./g. in October, and Danish butter (48) from 10·8 I.U./g. in March to 56·7 I.U./g. also in October. The highest value quoted is 7 I.U./g. for May Jersey milk of unstated fat content (53). This last figure can be contrasted with the chemical estimate of 1 μg. of vitamin A and 5 μg. of carotene per 100 ml. of winter Belgian milk (43).

Much work has been devoted to the conservation of carotene in the fodder and its transference to milk and butter. In the United States ensiling of lucerne and other roughages by the A.I.V. and molasses processes has been in the foreground (6,44,54,55,56,57,58,59), and further studies are reported from this country (16) and by Virtanen himself from Finland (47). Artificially dried grass has also attracted attention, mostly in England (9,16,60,61,62). Among other products investigated were ordinary silage (16,56,57,58,59,63), carrot-maize silage (57,64), kale and sprouted maize (9), guinea grass (65), and special hay (66). The effect of feeding a vitamin A concentrate to cows was also studied (67). A review of the literature on grass silage has been published (68). From the standpoint of the vitamin A and carotene content of milk, molasses and A.I.V. silage made from lucerne were equal (6). Biological methods of assay were used in (44), (54), (56), (62), (63), (66) and (67). Marked differences are again reported. Thus Archibald & Parsons (67) reported milk potencies of 2700 I.U. per American quart on a daily intake of 90 mg. of carotene and of 3600 I.U. on the same intake of carotene supplemented with 32,000 I.U. daily of vitamin A. This corresponds to some 75–100 I.U./g. of butterfat. Hodgson *et al.* (63), however, obtained butter containing 50 I.U./g. on an intake of 185 mg. of carotene, and ten times this intake

yielded butter of twice the potency. The efficiency of transfer of vitamin A activity from food to milk can be calculated from the data of Hodgson *et al.* (63) at from about 7% for low in takes to about 1% for higher intakes. Archibald & Parsons (67) quote values of from 0·3 to 2%; a calculation, however, from data given in the paper yields the improbable figures of from 5 to 30%.

Moore (69) studied the carotene content of the blood plasma of different breeds of cows kept under similar conditions of feeding and found that the concentration increased in the order Brown Swiss, Holstein, Ayrshire, Jersey and Guernsey, i.e. in the order believed to represent gradations of butter colour.

Sharp & Hand (46) examined the carotenoid content of butter from the cows of different breeds composing the herd of the Cornell Experiment Station and found that this increased in the order Holstein (*20*), Ayrshire (*11*), Brown Swiss (*3*), Jersey (*25*) and Guernsey (*13*). The italicized figures in brackets indicate the number of cows examined. The mean values for Holsteins were 3·5 mg./l. during stall feeding and 5·8 mg./l. on pasture. The corresponding figures for Guernseys were 8·5 and 16·4 mg./l. respectively.

A rapidly increasing volume of work is devoted to the question of oxidized flavour in milk. Most authors agree that milks of high carotene content are less likely to develop the fault (57, 58, 59, 70, 71, 72, 73, 74), though no difference in this respect was found by Guthrie (75) between milks produced on winter and summer rations and presumably differing markedly in carotene content. The effect is apparently a true carotene effect, as the appearance of oxidized flavour is prevented not only by access to pasture and to roughage of high carotene content but also by carotene concentrates (73, 74). In agreement with this, Beck *et al.* (71) found Jersey and Guernsey milk less susceptible to the formation of the taint than the milk of Holsteins or Ayrshires. Vitamin A has almost no protective action (74), and the feeding of cod-liver oil caused off-flavours to appear (75).

Brouwer *et al.* (76) have studied the effect of the Hofius method of preservation of milk under high oxygen pressure on vitamin A and carotene and found that there was little loss in milk and cream kept in stainless steel containers for from 1 to 3 months under an oxygen pressure of 10 atm. at 4–5 and also at 19° C. Carotene was more easily affected than vitamin A. The loss was greater in tinned copper. According to Olson *et al.* (18) freezing of milk has no effect on vitamin A and carotene.

Henry *et al.* (77) obtained duplicate samples of fat from fourteen samples of milk by churning and by extraction with solvents. On the assumption that there was no loss of carotene or of vitamin A in the extraction they found that churning had caused a slight loss of carotene but no change in the vitamin A content. Sherman & Sherman (78) have collected from the literature data for the vitamin A activity of fifty-three samples of milk and of eighty-six samples of butter and conclude from the comparison that the recovery in butter of the vitamin A activity of milk is short of completeness by some 10–15%. As the butters and milks were from different sources, and as some tests were carried out on butter and others on butterfat, the significance of the difference seems questionable. In any case, without knowledge of yields a comparison of milk and butter values cannot be used as a measure of completeness of recovery. As a measure of destruction in the course of handling, a figure of 10–15% is probably too high.

The vitamin A and carotene content of colostrum or colostral fat has been studied by Kramer *et al.* (79), Gillam & El Ridi (32), Stewart & McCallum (80) and Henry *et al.* (81). Biological tests (79) agreed with chemical and physical measurements (81) in showing that the first colostrum is some 20 times as potent as later milk from the same cow. Access to pasture before calving appeared to have no effect on the secretion of vitamin A but increased the output of carotene in colostrum (81). The

extensive study of the colostrum of 100 cows in Scotland (80) showed a very wide range of values, the ratios of the highest to the lowest vitamin A figure being 22 : 1, and 40 : 1 for carotene. The marked difference in vitamin A values between papers (80) and (81) is possibly due to differences in the technique of carrying out the antimony trichloride test.

Grewal & Kochhar (49), De & Majumbar (82) and Banerjee (83, 84) studied the vitamin A content of ghee. Figures for the vitamin A content of milk in Batavia have been published (85).

(iii) *The vitamin B complex.* The nutritional importance of riboflavin for man has been clearly demonstrated (86, 87). On the other hand, the striking claim that riboflavin phosphate can function as a separate vitamin, reported in the previous review (2), has not been substantiated (88).

The thiochrome test of Jansen (89) has been applied to the estimation of vitamin B_1 in milk by several authors (90, 91, 92, 93, 94, 95, 96, 97, 98), in some cases with little modification and mostly without simultaneous biological check. It is now known that the test in its original form cannot be applied to milk (92, 93, 94, 95), and in consequence the results of Widenbauer & Heckler (97), and regrettably also the painstaking investigations of Wiegand (90, 91), are of little practical value. For milk Jansen's test must be modified to measure not only "free" but also phosphorylated and protein-bound vitamin B_1 (94, 95), and this is best achieved by treatment with takaphosphatase and pepsin (94, 95, 96, 98). There is a marked correlation in milk between the content of "free" vitamin B_1 and the phosphatase (phosphomonoesterase) titre. Colostrum, with the exception of the first day's, and early milk are low in phosphatase and contain much cocarboxylase, while late lactation milks high in phosphatase contain little or no phosphorylated vitamin B_1. The total vitamin B_1 content of milk is independent of the feeding but changes with lactation from some 60–100 μg./100 ml. for colostrum and early milk to 30–35 μg./100 ml. for mid-lactation and 30–40 μg./100 ml. for late lactation milk (95). Daniels (99) assayed milk by a biological method, and an indirect calculation suggests that 1 ml. was equivalent to 1 μg. of crystalline vitamin B_1, a very high value. Vahlteich (100) measured the amount of vitamin B_1 and riboflavin present in the aqueous phase of butter and margarine and found respectively 26 and 14 μg./lb. of vitamin B_1 and 38 and 32 Bourquin-Sherman units per lb. of riboflavin. (A Bourquin-Sherman unit is equivalent to 3–5 μg. of riboflavin (100a).)

Various modifications and improvements of the fluorimetric or colorimetric estimation of riboflavin in milk and milk products have been reported (101, 102, 103, 104, 105, 106). In biological tests the Bourquin-Sherman method, discussed in the previous review (2), (103, 107, 108) and other methods (9, 104, 109, 110) have been used. It is generally accepted that the agreement between biological and chemical methods of estimating the riboflavin content of milk is good, though comparisons have seldom been direct (106, 110). A microbiological test for riboflavin based on the growth response of *Lactobacillus casei* has also been described (111). It is said to agree well with chemical and biological tests (106, 111).

Fluorimetric tests show that there is a seasonal variation in the riboflavin content of milk which in summer contains some 20–50% more of the factor than in winter (95, 112, 113, 114, 115), and there is good evidence to show that the increase is due to pasture (95, 112, 113); cows having access in winter to phosphoric acid silage maintained a high level throughout the year (112, 113). The difference in riboflavin content between pasture and winter milk is less marked than in vitamin A and carotene content, but it is not yet clear whether the disparity between the content in the fodders is less or whether the cow is less dependent on exogenous sources for riboflavin than for carotene. The evidence from biological assays is less definite (9, 108), and it is suggested that climatic and seasonal factors may be of more importance that the diet (108). It is

generally agreed that Channel Island cattle produce milk richer in riboflavin than that of other breeds (108,113,115), the order being Jersey > Guernsey > Ayrshire > Holstein. The average values in μg./100 ml. quoted by Hand & Sharp (113) were for stall and pasture feeding respectively (italicized figures in brackets indicate the number of cows tested): Jersey (*25*, *24*), 218, 237; Guernsey (*14*, *14*), 191, 204; Brown Swiss (*4*, *5*), 187, 237; Ayrshire (*9*, *10*), 169, 197; Holstein (*22*, *23*), 142, 164. Whitnah *et al.* (115) placed Holsteins above Ayrshires. These authors claim that riboflavin content is positively correlated with the milk fat and that riboflavin may have a role in the formation of milk fat, but this is denied by Hand & Sharp (113).

Early colostrum contains some three times as much riboflavin as later milk (95,107). Some 10% of the riboflavin present in milk is in a bound form (95,114). A flavo-protein which catalyses the oxidation of hypoxanthine, aldehydes and dihydrocoenzyme I has been isolated from milk (116,117,118,119).

Dorfman *et al.* (120) have used the growth of the dysentery organism on a synthetic medium for the estimation of nicotinic acid in milk. Further assays of this factor are described on p. 209.

(iv) *Vitamin C.* A good review of the literature on vitamin C has been written by Cimmino (121), and a compilation of data on the vitamin C content of milk will be found in articles by Kroker (122) and Glickstein & Frandsen (123).

Several new or modified chemical methods of estimation of vitamin C have been published (124,125,126,127,128,129,130,131). They deal, with one exception (131) only, with the determination of the reduced form of ascorbic acid, but method (130) was specifically designed for milk as it comes from the cow, which normally contains no oxidized ascorbic acid. The values for milk and milk products quoted in (127) are either wrongly calculated or the method is grossly at fault. According to Bezssonoff & Vertruyen (132) ascorbic acid oxidase cannot be used for the estimation of the factor in milk. Van Eekelen (133) has critically reviewed methods for the assay of vitamin C.

Values for the vitamin C content of milk, mostly market milk, are available from the United States (134,135,136,137,138,139,140,75,131,71,141), Italy (121,142,143), Germany (122,144), Switzerland (145), France (146), Rumania (147), Greece (148) (abnormally high values), Russia (149), Norway (150), Finland (151), England (9), the Philippine Islands (152), Java (153) and Japan (154). It is surprising that the estimation of both forms of ascorbic acid is still the exception rather than the rule (9,121,122,131,150,154), especially as, in addition to those just quoted, other authors (136,137,138,140,146,151) well realized the effects of exposure to light. While tests for reduced ascorbic acid obtained shortly after milking on milk protected from light (136,138,75,131,147,143) are probably only slightly below the total vitamin C content of the milk, they are of comparatively little value in gauging the antiscorbutic potency of market milks which contain large and variable quantities of the reversibly oxidized form. Mathiesen (155), for example, found that a biological assay of milk yielded values one-third to one-half higher than a chemical titration measuring only the reduced ascorbic acid. The finding that the normal healthy cow secretes the whole of the vitamin C in the reduced form (2) has been confirmed (121,130,131), and von Wendt (151) reports that in streptococcal mastitis the affected quarter or quarters may secrete hardly any vitamin C though the milk volume is not affected. He is of the opinion that the vitamin C of milk is synthesized in the udder. It is now generally believed that the vitamin C content of milk is independent of the feed, and this view is supported by many workers (75,122,136,141,150). Riddell & Whitnah (156) have shown that when the intake of vitamin C is raised there is a temporary rise in the blood level and a marked increase in the output in the urine, but no increase in the milk. Similar results were reported by von Wendt (151) after intravenous injections of ascorbic acid. A peculiar indirect dietary effect was reported by Guthrie (75) who observed marked increases in the vitamin C content of milk

(60% on the average) when cows were given up to 400 ml. daily of cod-liver oil. There is less agreement concerning seasonal fluctuations of the vitamin C level. Some authors (136,149) found no differences, several others (138,140,144,150) noticed a peak in the winter months, one (139), on the contrary, obtained lowest values in winter, and one (142) reported differences which are not borne out by the experimental data. Breed differences have been observed (71,131,135,138), and there is agreement that Guernsey milk is richer than Holstein.

The relation of vitamin C to off-flavours in milk has been studied, but opinions are less unanimous than in the case of carotene. Certain authors (70,73,139,144) recorded a protective action of ascorbic acid, others (71,141) denied any correlation. According to Guthrie *et al.* (136) the destruction of vitamin C and the development of oxidized flavour are due to the same causes.

The opinion expressed in the previous review (2) that riboflavin acts as a sensitizer in the destruction of the vitamin C of milk by visible light has been confirmed by the experiments of Hand *et al.* (157). They also confirmed the finding that no destruction takes place in the absence of oxygen and describe an ingenious method of freeing milk of dissolved oxygen and also its application to market milk (157*a*). Ultra-violet light, on the other hand, causes losses even under anaerobic conditions (158). It has been reported (159) that the vitamin C of mare's milk which contains but little riboflavin is much less sensitive to the action of light, but human milk is rapidly affected (160). As would be expected (2), brown glass bottles exert a marked protective effect (122,161), green bottles are of less value (161). Cartons, though less effective than either, are decidedly better than clear-glass bottles (136,161).

The addition of ascorbic acid to milk has been suggested (122,143), and a natural increase of the factor in the course of lactic acid fermentation has been reported (149).

A method for the estimation of the solubility in milk of copper from copper-nickel alloys based on the catalytic effect on the oxidation of vitamin C has been described (162).

Citrin (vitamin P), which is said to accompany vitamin C in certain natural products, could not be detected in milk (163).

(v) *Vitamin D.* The seasonal variation of the vitamin D content of milk has been studied in this country (48) and in the United States (164). Qualitative tests were done by Depetris (165) in the Argentine. The extensive series of observations of Wilkinson (48) on Scottish and Danish butters shows clearly the sharp summer peak of vitamin D activity and its correlation with the amount of ultra-violet light received by the cows. The highest, lowest and mean values in I.U./g. were for Scotland 0·99 (June), 0·08 (March, April, December), 0·32; and for Denmark 0·54 (July), 0·08 (December, March, November), 0·23. Wallis & Olson (164) obtained similar values: 0·23 in April, 0·83 in July and 0·28 in November. Their estimations were done on butterfat. These authors also believe that the summer sunshine is largely responsible for the increased vitamin D content of summer milk. A similar view is expressed by Olson (166) and is supported by the experiments of Wallis (167). The effect of diet was studied by Wallis & Olson (164), Wallis (168,169) and Salisbury & Morrison (66). Six Holstein cows were kept on a vitamin D-deficient ration for 2 months, and at the end of this time a pooled sample of butterfat had a potency of 0·19 I.U./g. The cows were then divided into three groups of two, of which one was continued on the deficient ration, another was given daily 10,000 I.U. of vitamin D as alfalfa hay, and the third half this quantity as prairie hay. The activity of the butter of the first group became too low to be measured by the line test technique, that of the second group rose to 0·46 I.U./g. in three months and that of the third group changed but little. Between 1 and 2% of the vitamin D of the feed was recovered in the butter (164,169). Wallis (170) gave a Holstein and a Jersey cow the same amount of vitamin D (approximately 19,000 I.U.

daily) throughout lactation. Though the vitamin content of the Jersey milk fluctuated between 27 and 33 I.U. per American quart and that of the Holstein cow between 8 and 12 I.U., the total vitamin D output in the milk was about the same for the two cows. The recovery in butter amounted to from 0·5 to 1·8% of the intake. Van Niekerk & Bliek (171) report that colostrum collected during the first 24 hr. has 6–10 times the activity of normal milk obtained at the same time of year.

The failure to concentrate quantitatively the antirachitic activity of butterfat by saponification or extraction with alcohol, which was mentioned in the previous review (2), has been amply confirmed (48,168,169,172,173,174) and similar results have been obtained with the fat of human milk (175). The explanation of the phenomenon given by Gridgeman *et al.* (174) is erroneous, and Henry & Kon (176) have now proved that this is due in the rat test to the antirachitic effect of fat, quite distinct from that of the vitamin D present in butter. They suggest that fatty substances of low antirachitic potency such as butter and margarine should be saponified before testing. Morgareidge & O'Brien (177,178) have studied the "enhancement effect" when vitamin D is dispersed in milk (2) and have shown that, for rats, this is due to the phosphorus present in the milk. They point out that human milk contains only about one-fifth to one-sixth as much phosphorus as cow's milk, and that this may account for the very low apparent vitamin D content reported in the literature for breast milk. It is of interest in this connexion that Drummond *et al.* (179) measured in careful experiments the vitamin D content of fat isolated from human milk. They reported that the mean values for the antirachitic activity of the milk of mothers receiving diets decidedly low in the factor were equal to those of cow's milk in summer and found that mothers who took more vitamin D produced milk twice as active.

(*c*) *Other factors*

(i) *Lactose.* Outhouse *et al.* (180) have continued their studies on the effect of lactose on the assimilation of minerals. They suggest that it stimulates primarily the storage of calcium and that the effect on the retention of phosphorus is less marked. Mitchell *et al.* (181) found that calcium or sodium gluconate inhibited the absorption of lactose from the alimentary tract, probably by affecting its breakdown to simple sugars. The factors concerned in and the mechanism of the production of experimental lactose (galactose) cataract have been studied by a number of authors. Increasing the level of protein in the diet has an inhibitory action (182,183,184,185), but neither riboflavin nor aneurin in massive doses were effective (186,187,188). According to one author (189) galactose lowers the permeability of the lens, but exactly the opposite is stated by another (190). The cataractous changes regress when the feeding of galactose is discontinued (182,184,191), mostly owing to the growth of fresh fibres at the periphery of the lens. When fed in equivalent amounts galactose is decidedly more toxic than lactose (184,187). It is suggested (187) that this is because the hydrolysis of lactose in the gut is sufficiently slow for the liberated galactose to be completely metabolized. It should be remembered, however, that according to Herrington *et al.* (192) lactose may be hydrolysed by the rat to a very appreciable extent within 1 min. of ingestion. Cashell & Kon (184) found that lactose as present in dried skim milk is as toxic as that in synthetic rations, and that the feeding of fresh or reconstituted liquid separated milk is definitely more injurious to the lens than the feeding of the milk in the dry form. They produced mature cataracts on liquid milk diets. Krewson *et al.* (187) reported no cataract formation on skim milk, but by their technique only mature cataracts would be detected. Papers (193), (194) and (195) also deal with galactose cataract. Schantz & Krewson (196), in further studies of the effect of fat on the utilization of lactose from skim milk, found that even-chain fatty acids containing less than 12 carbon atoms and synthetic odd-chain fatty acids were without effect,

but that longer even-chain acids were effective in preventing the loss of galactose in the urine.

(ii) *Soft curd milk.* The subject has been reviewed in several papers (197, 198, 199, 200, 201, 202) of which the extensive review by Doan (197) should be specially mentioned. New ways of preparing soft curd milk are described in (203) and (204), and various methods used for *in vitro* estimation of the digestibility of different types of such milks are given in (202), (205), (206), (207) and (208). Judging by the disparity of the results, there is little doubt that the different techniques do not measure the same property or properties of the milks. Similarly no unanimity is reached in *in vivo* experiments (203, 204, 208, 209, 210). That soft curd milk is of value in human nutrition is discussed later in this paper. According to Woessner *et al.* (131) "mineral modified" milk is low in vitamin C, a fact which is not surprising in view of the treatment to which the milk is exposed.

(iii) *Minerals.* The significance of cobalt in nutritional anaemia has been investigated by Elvehjem and his collaborators. In the rat optimal haemoglobin regeneration on a milk diet was obtained in the presence of iron, copper and manganese alone, and the addition of cobalt was without effect (211). In the dog, however, recovery from milk anaemia was slow, and a plateau was reached at about 10–12 g. of haemoglobin per 100 ml. blood unless cobalt was added, which made complete regeneration possible (212, 213). Kohler *et al.* (214) have shown that chlorophyll, protoporphyrin and bilirubin, which might act as haematin precursors, could not be built up into haemoglobin in the presence of optimal amounts of iron when copper was absent. The recovery from milk anaemia brought about by iron and copper can, however, take place normally in the absence of the spleen (215).

Smith & Otis (216) have shown that the feeding of copper alone to rats previously rendered anaemic by a milk diet mobilizes the body stores of iron and causes some haemoglobin formation, and though this is denied by Harris & Poland (217) it is in agreement with the recent findings of van Donk *et al.* (218). To deplete completely the copper stores of experimental animals Frost & Elvehjem (213) feed milk and pure iron followed by milk alone, and Summerson (219) advocates treatment of milk with H_2S to speed up the production of copper deficiency. Alt (220) found that the young of rats whose iron reserve had been depleted by producing a previous litter on a milk diet were anaemic at birth. According to Lucas & Summerfeldt (221) ferrous chloride was more effective in conjunction with copper in curing milk anaemia in rats than ferric salts. In this connexion it should be noted that Frost *et al.* (222) obtained cures in dogs maintained on exclusive milk diets with exceptionally small quantities of ferric chloride. Further information on the role of iron, copper and cobalt in milk anaemia will be found in other papers (223, 224, 225, 226).

Whole milk supplemented with iron, copper and manganese has been used by Hart, Elvehjem and collaborators as a convenient diet for the study on rats of the nutritional importance of several elements. It was found that no improvement could be detected when aluminium (227), cobalt (211) and arsenic (228) were added to such diet. These elements are either not essential for the rat or the requirement is respectively less than 1, 0·6 and 2 μg. per day which are supplied in the daily milk ration. An indication of a beneficial effect was obtained with boron (229). Cow's milk is relatively low in magnesium, but it has been shown by Day & Orent-Keiles (230) that it contains enough to cover the requirements of rats receiving whole milk diets supplemented with Fe, Cu and Mn. The effects on rats of additions of Cu, Fe, Zn, Mn, Ni and Co to exclusive milk diets are discussed in (231). Black *et al.* (232) have shown that rats receiving a supplement of iron and copper grew better and stored more energy, nitrogen and fat than littermates which had equal quantities of milk alone. Schwartzer (233) kept rats on milk alone in special collars which completely prevented

coprophagy and found that they survived only half as long as rats allowed access to urine and faeces.

(iv) *Milk proteins.* A full report has appeared (234) of the work of Kik on the nutritive value of lactalbumin mentioned in the previous review (2). Greaves *et al.* (235) have studied the effect of heat treatment on the biological value of casein. They found that heating for 8 hr. at 100° C. was without effect, but that at 130° C. perceptible injury was produced in 30 min. They express the opinion that lysine is the first amino acid to be damaged by heat and histidine the second, and rightly point out that when heat damage to a protein is assessed by adding to it different essential amino acids and measuring the biological value with and without supplement the same procedure should be applied to the unheated protein. The benefit derived from the added amino acid need not necessarily indicate that this amino acid had been injured by heat; it may equally well show that it was a limiting factor in the original protein.

Casein is extensively used in "synthetic" diets, and the complications have been discussed arising from contamination with vitamin A and carotene (236), riboflavin (237, 238), the chick anti-dermatitis factor (238) and fluorine (239). Lilly (240) noticed a marked decrease in the incidence of dental caries in rats when casein replaced whole-milk powder in a diet, the other constituents of which were maize meal, linseed cake, lucerne meal and NaCl.

(v) *Milk fat.* Schantz *et al.* (241) incorporated by homogenization into separated milk containing only 0·04% fat, 4% respectively of butterfat, maize oil, coco-nut oil, cotton-seed oil and soya-bean oil. The essential minerals and carotene were also added and the mixtures were fed to rats as the sole diet. Vitamin D was supplied by irradiating the animals. The butterfat group grew better and fared better than rats receiving the vegetable oils. This superiority was connected with the fatty acids fraction of butter and not with the non-saponifiable residue. On the other hand, Sherman (242) found that soya-bean oil and linseed oil and to a lesser degree maize, cotton-seed and wheat-germ oils enhanced the growth effect of carotene and cured scaliness in vitamin A-deficient rats suffering from fat deficiency disease, while butterfat and coco-nut oil were inactive in both respects. Hartwell has studied *in vitro* the digestion of reconstituted cream (243) and margarine (244). The nutritive value of margarine is discussed in two further papers (245, 246). Nicolaysen (247) found that in young rats receiving a diet rich in vitamin D and of moderate calcium content the utilization of this element was unaffected by the presence or absence of butter or other fats. Euler *et al.* (247*a*) noticed similar retentions of calcium by rats whether the dietary fat consisted of butter or of margarine. A method for the detection of annatto in butterfat is described by van Duuren (248).

B. Treated milk

(*a*) *Heated milk*

(i) *General.* The nutritive effects of the pasteurization of milk have been extensively reviewed (249, 250, 251, 252). In addition a summary of the results of investigations carried out in this country on behalf of the Milk Nutrition Committee has now been published (253).

By the technique of exclusive milk feeding (cf. p. 197), Bartlett *et al.* (10) were unable to detect any deleterious effect of holder pasteurization on the growth-promoting value of winter or summer milk produced under south of England conditions. The same result was obtained when cane sugar was added to the milks and the caloric value doubled.

Anderson & Elvehjem (254) found that milk supplemented with minerals and

vitamin D is a satisfactory diet for dogs. Females produced litters on this regimen which throve equally well whether the milk was raw or pasteurized.

The experiment on calves mentioned in the previous review(2) has now been published. The report(255) contains not only the details of comparisons between raw and pasteurized milk carried out in Reading and Aberdeen but a critical survey of information available from other sources. The results of the new experiments agree with the conclusions which can be derived from the whole body of evidence and show that if pasteurization had any deleterious effects these were of a quite minor kind. Most of the experiments on calves have been complicated by tuberculosis, chiefly in the raw-milk groups, and an investigation from which this complicating factor would be excluded would be of considerable interest.

Andross(256) has measured the loss of nutrients in the deposit left on the pan and in the skin which forms when milk is boiled under household conditions. Over 10% of the total solids may be lost in this way.

(ii) *Vitamins.* Kroker(257) has summarized the information on the effect of heat treatment and storage on the vitamins of milk, and a most useful compilation of data on the effects of pasteurization and boiling has been published by Fixsen(258).

Deco(259) studied by Lovibond Tintometer tests the effect of heat treatment of milk on vitamin A and carotene. Pasteurization and ordinary boiling were without effect, but prolonged (75 min.) and sometimes repeated boiling to which milk was subjected in a Liège hospital led to the loss of as much as half of the original vitamin. Houston *et al.*(114) found by chemical methods that commercial pasteurization destroyed some 10% and commercial sterilization about 50% of the vitamin B_1 of milk but that neither treatment affected the riboflavin. Hand(102) also found that the latter withstood pasteurization without loss.

A considerable volume of work has been published on the fate of vitamin C in the course of pasteurization(121,122,131,134,136,137,139,140,143,145,146,147,150,154,157,157*a*,260), but for reasons already stated on p. 202 much of it is unsatisfactory. In the more careful experiments(121,131,137,140,154) losses of 35, 27, 30, 19 and 19% respectively have been reported, but according to one author(150) the loss in high temperature-short time pasteurization in a plate pasteurizer was negligible. Similarly, very small losses were reported from Denmark for H.T.-S.T. pasteurization in the Silkeborg Stassano outfit(261), the "Spirala"(262), "A.P.V."(263) and "Kolding"(264) pasteurizers. Dehydroascorbic acid was more affected by pasteurization than the reduced form(131,154). The relation of vitamin C to oxidized flavour in pasteurized milk is discussed in several papers(136,139,157,157*a*). According to the last two, milk freed from oxygen can be pasteurized without loss in the presence of copper. Pratt(265) found that an atmosphere of CO_2 exerted a protective effect.

(*b*) *Irradiated and vitaminized milk*

Weckel & Jackson(266), writing from first-hand knowledge, have published a most valuable review which deals with all aspects of the formation of vitamin D in milk by ultra-violet irradiation. Beck *et al.*(267) have studied the factors affecting the anti-rachitic response, while O'Brien *et al.*(268) have shown that activation of milk up to the strength of 2 I.U./g. is theoretically possible and have achieved in practice potencies of 1·6 I.U./g. Methods of producing irradiated milk of high vitamin D potency are also discussed by Dorcas(269), and commercial irradiators capable of producing milk containing 400 I.U. of vitamin D per American quart (i.e. the maximum recognized by the Council on foods of the American Medical Association(270)) are described in(271) and(272).

Methods adopted by various bodies in the United States for the assay of vitamin D

milks have been described (273, 274, 275, 276). Attempts to concentrate quantitatively the antirachitic activity of vitamin D milks have been unsuccessful (277, 278, 279).

Fleisch (280) showed by experiments on chicken that the antirachitic potency of irradiated milks is due not to vitamin D_2 but to another substance, probably vitamin D_3. He found that the vitamin D of irradiated milk was more efficiently utilized by chicks than a solution of vitamin D_3.

Van Niekerk & Hofstra (281) fed to cows (according to their milk yield) from 75,000 to 90,000 I.U. daily of a vitamin D_3 concentrate, and increased the vitamin D content of the milk from 20 to 60–80 I.U./l. About 1·7% of the vitamin D fed was recovered in the milk.

The effect of irradiation of milk on vitamin C and carotene has also been discussed (282, 283, 284). Homogenization, which forms part of the process of enriching milk with vitamin D by addition of vitamin D concentrates, has a detrimental effect on vitamin C (131). The publication *Vitamin D Milk* and the new publication *Vitamin D Digest*, both issued by Standard Brands, Inc., of New York, contain useful summaries and lists of references.

C. Milk products

(*a*) *Cheese and whey*

Orru (285) studied on rats the value of ricotta (a type of albumin cheese made from whey) as a supplement to wheat flour, and Palmer (286) the value of bread-cheese combinations. Qualitative tests of the vitamin content of fresh, pasteurized and ripened cheese are reported by Balsamelli (287). Hathaway & Davis (288) estimated roughly the vitamin A activity of twenty-four varieties of cheese and found highest values for Roquefort and Camembert and lowest for Cottage, Neufchatel and Limburger. The Cheddar tested by them contained about 20 I.U./g. For a cheese of the same type made in March, Guerrant *et al.* (289) quote a value of 10 I.U./g., while Davies & Moore (290) report 7·5 I.U./g. for English Cheddar. No loss of either carotene or vitamin A was detected when Emmenthaler cheese, 3–4 months old, was compared with the original milk (291), and only a negligible loss of vitamin A activity throughout ripening and storage of Cheddar in the course of one year (289). Figures for the vitamin B_1 content of eighteen types of cheese are given by Pyke (98).

Jukes & Richardson (292) studied on chicks the concentration of riboflavin and of the filtrate factor in various types of dried milk, dried whey and buttermilk and found that the latter was definitely richer in riboflavin than skim milk. A similar observation was made by Strong & Feeney (293), who report a content of about 35 μg./g. for dried buttermilk and 17–21 μg./g. for dried skim milk and dried whey. Hodson & Norris (106) quote values of from 18 to 26 μg./g. for the riboflavin content of dried whey. Supplee (294) discusses the value of whey as a source of vitamins and describes briefly the preparation from it of a vitamin concentrate containing vitamin B_1, riboflavin, vitamin B_6, vitamin K, provitamin D and the oestrogenic hormone.

Autoclaved whey has been used as a source of the vitamin B_2 complex in vitamin B_1-deficient diets for rats (99, 295), but later work (296, 297) showed that it did not supply all the members of the complex.

(*b*) *Dried and evaporated milk*

Henry *et al.* (101) found no difference in the growth-promoting value of spray- and roller-dried and of evaporated milk made from one batch of raw milk when these were fed to rats *ad libitum* as the sole diet, supplemented with Fe, Cu and Mn. The gains on evaporated milk were, however, less economical. In experiments in which

only Fe and Cu were added to milk Valenzuela (298) obtained less good growth on "a well-known brand of reconstituted milk which is known to be made from milk constituents that have undergone severe heat treatment" than on raw or pasteurized milk.

The improvement in the nutritive value of bread brought about by the addition of dry skim milk to the flour is clearly demonstrated by the thorough research of Fairbanks (299,300). Forty-four generations of rats have been reared on a simple diet of one part whole-milk powder, five parts ground whole wheat and 2% NaCl (301), and the effects of increasing its Ca content (301) or of the addition of meat and green vegetables to a diet similar but containing one-third of whole-milk powder (302) have been studied. The addition of skim-milk powder to a "poor Madrassi diet" was without effect on the assimilation from it of carotene by rats (303).

Hutchinson (304) compared on cats the digestibility of fat, protein and ash of raw milk with those of Australian dried whole milk and found little difference. He also quotes figures for the vitamin content of the product.

Henry *et al.* (101) found no loss of vitamin A and carotene in the manufacture of roller- and spray-dried and of evaporated milk, and similar conclusions are reached by Meulemans & de Haas (305). Fleisch & Schnieper (306) believe, however, that evaporated milk has a lower vitamin A content than sweetened condensed and dried milk. This difference was probably fortuitous as the milks were of different origin, and no decrease in vitamin A was observed when raw and evaporated milk from one bulk were compared. Houston & Kon (307) observed only a small loss of vitamin B_1 (i.e. about 10%), in the spray-drying of milk, but Henry *et al.* (101) found evaporation much more injurious, evaporated milk containing only half the vitamin B_1 present in spray- or roller-dried milk prepared simultaneously from the same batch of raw milk. According to Daniels (99) the loss in evaporation is 60%, a very similar figure, and Jao *et al.* (308) report an even higher loss, 75%, for sweetened condensed milk; their methods, however, allow of only a rough estimate. Schlutz & Knott (296) observed much smaller losses of vitamin B_1, from 20 to 34%, in the evaporation of milk, but noted a gradual loss on storage. Pyke (98) quotes values of 32, 19 and 85 I.U./100 g. obtained by the thiochrome test respectively for sweetened condensed, evaporated and dried skim milk.

Houston & Kon (307) detected no loss of riboflavin in the spray-drying of milk, and the values quoted for dried skim milk by Hodson & Norris (106) and Snell & Strong (111), amounting to 17–22 μg./g., certainly support this view. Levine & Remington (309) also quote figures for the riboflavin content of dried milk.

McKibbin *et al.* (310) assessed in biological tests on dogs the nicotinic acid content of dried skim milk at from 0·05 to 0·15 mg./g. Swaminathan (311) obtained by a chemical method a similar value, 0·1 mg./g.

The loss of vitamin C in spray-drying and in roller-drying and evaporation is put by Henry *et al.* (101) at 20 and 30% respectively. There was also subsequent gradual loss in the evaporated milk. Meulemans & de Haas (305) report, on the contrary, no loss for evaporated milk and a 50% loss for dried, sweetened condensed, and separated sweetened condensed milks, but their estimates are not based on direct comparisons of milk before and after processing. Losses of reduced ascorbic acid during evaporation of milk have been observed by Fleisch & Schnieper (306), these being more marked when the final sterilization was carried out in the presence of traces of air than in an atmosphere of nitrogen. Schmid (312) has investigated the vitamin C content of an impressive collection of evaporated, sweetened condensed and dried milks of Swiss, German, Austrian, French, Italian and American origin. Unfortunately only the reduced ascorbic acid was measured. He noticed a rapid disappearance of vitamin C after reconstitution of the milks. It is probable that this was due to exposure to light.

Vitamin D is remarkably stable in irradiated evaporated milk, there being hardly any loss in 2–3 years' storage (313).

Experiments on dogs show that vitamin E suffers some loss in the evaporation of milk (254,314).

The biological value of the proteins of processed milks has been studied by several authors. Kik (315) reports a true digestibility of 93·5 and a biological value of 85·6 for the proteins of whole milk powder at an 8% level of intake and 93·9 and 87·8 respectively for skim-milk powder. Sumner (316) working with young rats obtained for skim-milk powder a biological value of 84 at the same level of intake. For adult, 1-year-old, rats the value was distinctly lower, 64 at 8% and 78 at the 5% level of intake. Henry *et al.* (101) quote values of 80·4, 83·3 and 84 and true digestibilities of 90·9, 92·6 and 93·6 respectively for evaporated, roller-dried and spray-dried milk. Swaminathan (317,318,319) and Basu & Haldar (320) have demonstrated the beneficial effect of addition of dried milk to rice, ragi (*Eleusine coracana*) and to different pulses on the biological value of their proteins.

The question of the effect of heat treatment on the availability of milk calcium for rats is still controversial. Fairbanks & Mitchell (321) found that calcium from spray-dried separated milk was some 7% less available than calcium from raw liquid milk; they found, however, that the addition of ascorbic acid to the heated milk diet improved the utilization of the element. The latter findings were confirmed in a subsequent study (322) which, moreover, showed no difference in the utilization of Ca from raw or dried separated milk when ascorbic acid was added to both diets. The authors suggest (321) that the decrease in the availability of milk Ca is due to destruction of vitamin C in the drying. The experience of Henry & Kon (323,324) is at variance with these findings. They obtained on the one hand under favourable conditions almost 100% retention of the calcium of spray-dried whole milk (323) and on the other observed no difference in the assimilation of Ca from an inorganic source whether the diet was devoid of vitamin C or supplied 2 mg. of it daily (324). It should also be remembered that the vitamin C content of dried milk is only some 20% less than that of raw milk (cf. p. 209), and that Kung *et al.* (325) obtained 100% retentions of Ca from dried skim milk using diets which supplied little if any vitamin C other than that present in the milk. In their recent paper (322) Fairbanks & Mitchell also state that vitamin C is not the complete explanation, and that the calcium of dried separated milk may be as well utilized as that of the raw milk.

Lanford (326) observed a statistically significant improvement in the assimilation by rats of calcium from a whole wheat-dried milk diet following the addition of orange juice. Previous work indicated that such beneficial result might be due to the effect of citric acid and citrates.

The calcium of spray-dried milk is slightly but significantly more available to the rat than that of $CaHPO_4.2H_2O$ (323). Campbell (327) reported no difference in the utilization of Ca from milk and from $CaCO_3$, but the Ca content of the diets was too high for this type of test. Tisdall & Drake (328) obtained very similar retentions of Ca from whole-milk powder and from calcium phosphate, carbonate, chloride and lactate. The addition of extra milk to a human diet very low in this substance increased not only the total but the percentage retention of Ca by rats (328). Patwardhan & Chitre (329) have studied the Ca content of various tissues of rats receiving diets containing 21–28% of dried skim milk.

Watson *et al.* (330) observed lessened mortality in herds of mice exposed to natural contact infection with *Bact. typhi-murium* when dried separated milk was added to the diet. Dried winter milk was no less effective in raising resistance to infection than dried summer milk (331).

An unfavourable effect of addition of dried skim milk and of buttermilk to the

diet on the course of experimental caecal coccidiosis of chicks is reported by Becker & Waters (332).

IV. NUTRITIONAL VALUE OF MILK FOR HUMAN BEINGS

A. General

(*a*) *Introduction*

In the years under review the study of problems of human nutrition has been gaining further momentum, and an immense volume of work has been published. A large part of it has been devoted to dietary and economic surveys or has dealt with the production and distribution of food, but experimental studies have also been both numerous and extensive. The increasing realization by governments and responsible bodies throughout the world that the diet consumed by the bulk of the population is inadequate in kind even if sufficient in quantity has certainly stimulated investigation. In this field the Health Organization of the League of Nations has by its virile and inspired leadership achieved successes denied in other spheres to the main body of that Society. Paradoxically enough the tendencies of national self-sufficiency and isolationism exhibited by certain countries have also been responsible for considerable nutritional investigation.

(*b*) *Nutritional surveys*

Of the very many publications only a few can be mentioned here. The *Survey of National Nutrition Policies* by the League of Nations (333) contains much information on the consumption of milk, the improvement of supply and the distribution of cheap milk in various countries, and further data are available in two articles by McDougall in the League's *Health Bulletin* (334,335). The Technical Commission on Nutrition of the League of Nations has reported on the nutritive value of milk (336).

In this country the development of the Milk in Schools Scheme has been reported on (337,338), and the Conference on Nutrition and the Public Health convened by the British Medical Association has discussed the provision of cheap or free milk (339). The British Association for Labour Legislation states in its valuable and interesting *Report on Nutrition* (340) that "The low consumption of milk is the most significant of the signs of malnutrition throughout the country". Details of food budgets of nineteen working-class families in Ipswich (341) show how inadequate was the intake of milk and milk products. Similar conclusions were drawn from the study of the diets of 205 families in the West Riding of Yorkshire (341*a*).

The importance of milk in the diet of colonial peoples is stressed in Reports of a Committee of the Economic Advisory Council (342,343), and much information is given on the use of milk and its products in the colonies. Nutritional surveys of Australia (344,345,346) bring out clearly the connexion between malnutrition and a low intake of milk. In India a survey of 1500 children in Najafgarh in Delhi Province (347) showed a satisfactory nutritional status on a diet based on whole cereals and containing a fair quantity of milk.

In the United States an extensive investigation into diets of workers and their families in cities has been carried out (348). It contains useful information on the consumption of milk. An interesting comparison of the dietary habits and height of male students in New York City with those of their parents, of whom 70% were born abroad, showed that the sons were on the average 2·7 in. taller than the fathers (349). The difference was attributed to greater intake of milk and meat.

(c) *General effects*

The large-scale investigation into the nutritive value of milk sponsored by the Milk Nutrition Committee and mentioned in a previous review (1) has been completed, and an interim and a final report have been published (350, 253). Of over 8000 school children who volunteered for the experiment just over 6000 were present at all medical examinations. The children were derived from five areas, Luton, Wolverhampton, Huddersfield, Burton and Renfrewshire, and were randomly divided into four feeding groups: a control group receiving a small amount of biscuit as a *placebo*, a group receiving one-third of a pint of pasteurized milk, and two groups receiving respectively two-thirds of a pint of raw and of pasteurized milk. The heated milk was from the same bulk as the raw. The period of observation lasted for a year, and at the end of this time it was found that the milk supplements had brought about definite improvement in physique, in general appearance and scholastic ability and, to a lesser extent, in muscular strength. Two-thirds of a pint produced more marked effects than one-third of a pint, but the differences between raw and pasteurized milk were negligible. The gains in height and weight attributable to the milk were in this research definitely less than those observed by previous workers in this country. It is suggested that this was due to a general improvement in the diet of the people and to the smaller milk supplements used in the present study.

A number of smaller-scale studies of the value of milk supplements have been carried out in various countries. In the United States, Roberts *et al.* (351, 352, 353) investigated the effect of the addition of a second pint of milk to an institutional diet which already supplied approximately one pint (these were American pints, equal to 473 ml.). The supplement promoted gains in height and weight and produced better ossification of the bones of the wrist but was almost without effect on the spread of dental caries, which was very prevalent in the institution. Further reports come from Italy (354), the Philippine Islands (355, 356), Uganda (357, 358), the Nyasaland Protectorate (359) and North Borneo (360), and in most experiments the beneficial effects of milk were clearly evident though the groups of children were small and the time of observation relatively short. It is noteworthy, however, that grossly undernourished Bagishu Bantu children, mainly subsisting on a diet of cassava and millet, did not benefit from a daily addition of half a pint of boiled skim milk (358). In the Philippine experiment (355, 356) the children benefited more from the addition of reconstituted dried milk than from supplements of either soya-bean milk, cod-liver oil or tomato juice, though to the last three was added a salt mixture supplying Ca and P and other minerals.

The National Birthday Trust Fund has continued its scheme for improving the nutrition of expectant mothers in the special areas in England and Wales (361, 362). The relative importance, in lowering maternal mortality, of the milky foods and of other addenda ("marmite", vitamin and mineral preparations) supplied to the mothers is under investigation (361). A preliminary trial of "health dinners" (Oslo breakfasts) in a school in the poorest part of Stepney has clearly shown the importance of milk (363). Emergency diets for famine relief suggested by the League of Nations (364) consist of whole wheat-dried skim milk mixtures supplemented with cod-liver oil and salt.

B. Milk components

(a) *Vitamins*

Lewis & Barenberg (365) are of the opinion that in the average diet of artificially fed infants milk supplies more than enough vitamin A, i.e. probably four times the minimum requirement. Mackay (366), on the other hand, though she was unable to

confirm her earlier findings that babies receiving roller-process dried milk as the sole source of vitamin may be suffering from a slight grade of vitamin A deficiency, is still of the opinion that artificially fed babies should be given a vitamin A supplement.

The use in infant feeding in the tropics of sweetened condensed skim milk which "is ideal for producing in bottle-fed infants a vitamin A deficiency" (305) is universally condemned (305,367,368,369).

Virtanen (47) has calculated from surveys the amount of vitamin A derived from milk and milk products by the population of various parts of Finland.

(*b*) *Proteins*

Several studies of the assimilation of the nitrogen of milk by adults and children have been published (370,371,372,373,374,375,376). The biological value of milk proteins for adults is rather low, the estimates being 43 for liquid milk (373) and 62 for whole-milk powder (374). The latter value is much lower than those obtained on young growing rats, but little different from that given by adult rats (cf. p. 210). It is also very similar to the value, 65, obtained by the same authors (374) for egg proteins. It is rather surprising that definitely higher values were reported from the same laboratory for vegetable proteins (371); the calculations, however, involved in this case certain assumptions not necessarily correct. For premature babies percentage utilizations of 70·9 and 70·5 at a nitrogen intake of 2·75 g. nitrogen per kg. were obtained respectively for human and cow's milk (370). This did not include the allowance for metabolic nitrogen, and the biological value, if the necessary data were available, would presumably be higher than these already high utilizations.

Milk allergy in children is discussed in two papers (376*a*,376*b*).

(*c*) *Minerals*

The utilization of milk calcium has been chiefly studied and numerous papers have appeared on this subject. Valuable joint work has been published from the laboratories of Outhouse and of Mitchell in Illinois (377,378). This has shown that one (American) pint of milk would probably cover the Ca requirements of girls of pre-school age. The maximum daily retention of Ca was 7·5 mg./kg. body weight. The utilization of the calcium of dry skim milk by young children (boys and girls) amounted under optimal conditions to only 20% of the intake, a value very much lower than those observed on rats under similar conditions (cf. p. 210) but identical with the figure quoted by Steggerda & Mitchell (379) for an adult. It appears probable that children, like the growing rat, have no appreciable maintenance requirement for calcium (378). Souders *et al.* (376) observed Ca retentions on milk diets in children varying from 5·8 to 7·2 mg./kg. body weight daily, in agreement with the observations of Outhouse *et al.* (377). Pierce *et al.* (380) found that children utilized the calcium of $Ca_3(PO_4)_2$ as efficiently as the Ca of milk, and a similar observation was made by Steggerda & Mitchell (379) regarding the utilization of calcium gluconate by the adult. It will be recalled that rats also utilize inorganic calcium almost as well as the calcium of milk (cf. p. 210). Steggerda & Mitchell (379) found that a daily intake of 9·2 mg. Ca per kg. body weight was necessary for equilibrium in the adult, but much lower values have been reported from India (381), and Nicholls & Nimalasuriya (382) claim that tropical populations can adapt themselves to calcium intakes well below the accepted standards.

Gaunt *et al.* (383,384) analysed on rats the beneficial effects of the addition of milk and green vegetables to a poor human dietary (the Peterhead diet) and came to the conclusion that such effects were chiefly but not exclusively due to the Ca and P present in the addenda. Aykroyd & Krishnan (385) found that South Indian children

grew better when calcium lactate or liquid skim milk were added to their diet, but that they derived greater benefit from the latter.

McCance *et al.* (386) have studied the diets consumed by pregnant women of different income classes and found a marked correlation between the intake of milk and of calcium. According to Kramer & Gillum (387) women students in America are dependent on milk for adequate supplies of Ca and P. It has been pointed out by Jeans (388) that in childhood the need for calcium and the need for milk are more or less synonymous, and he puts the amount of milk needed to promote good bone growth between the ages of 4–6 years when growth is slow at a pint (473 ml.), and double that between 1 and 3 years and between 10 and 12 years. From 7 to 9 years 1½ pints is believed sufficient.

C. Effect of treatment

(*a*) *Effect of heating and processing*

The large-scale experiments of the Milk Nutrition Committee (253,350; cf. p. 212) demonstrated clearly the beneficial effect of an addition of milk to the diet of school-children. They showed equally clearly that in this respect pasteurized milk was just as good as raw. Few other papers have appeared which deal directly with the effects of heating and processing. Souders *et al.* (376) compared, in metabolic experiments on three healthy children 5–6 years old, liquid milk with plain evaporated and with evaporated irradiated milk. All three milks were obtained from the same herd of cows. There was a markedly better retention of nitrogen and an improved retention of calcium on the evaporated milks. Daniels (389) compared the mineral balance of children on pasteurized and on evaporated milk.

(*b*) *Irradiated and vitaminized milk*

Several authoritative reviews have appeared which deal with the problem of reinforcing milk with vitamins (390,391,392,393,394,395,396) or with the relative efficiency of the various vitamin D milks and other antirachitic agents (397,398). The article by Jeans & Stearns, mentioned in the previous review (2), has been published (399). As reported earlier (2) vitamin D_2 and vitamin D_3 and the various types of vitamin D milk are now believed to be equally efficient in the prevention and cure of human rickets. Fresh proof, however, is forthcoming to show that the menstruum has a definite influence, and that vitamin D dispersed in milk is more efficient than the same quantity given in oily solution (400).

There is general agreement that irradiated evaporated milk of the usual potency of 135 I.U. per American quart is very satisfactory for the prevention, though probably too weak for the cure, of rickets (397,398,401,402,403,404,405).

The value of irradiated fresh milk is emphasized by Scheer (406), while Grulee *et al.* (407) show that the impression apparently prevalent in the United States that irradiated evaporated milk causes diarrhoea is without foundation.

The metabolic studies on children of Souders *et al.* (376) and Hummel *et al.* (408) have demonstrated that when 400 g. of plain evaporated milk were replaced by 400 g. of irradiated evaporated milk there was an increase in the rate of gain of recumbent length, a more stable rate of formation of bone and a shift in the type of tissue formed from soft tissue to skeletal. It is of interest that in the studies of Roberts *et al.* (351, 352, 353) already mentioned on p. 212 no additional benefit was derived by the children from irradiated as compared with plain evaporated milk. The importance of milk, especially vitamin D milk, and of vitamin D therapy in the control of dental caries is discussed in two papers (409,410), but it appears that definitely higher levels of vitamin D are needed for this purpose than for the control of rickets.

(*c*) *Soft curd milk*

Very satisfactory results obtained in a home for infants from the feeding of raw undiluted milk homogenized by the Sonic oscillator method are reported(205). Reynolds *et al.*(411) followed by radiography the passage through the intestinal tracts of children of meals of pasteurized and evaporated milk and of milk treated with zeolite. All three milks were from one group of cows. It was found that the soft curd milks (evaporated and treated with zeolite) began leaving the stomach earlier than the pasteurized milk and that they formed in the intestinal tract fluffier and more evenly dispersed masses.

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